On the geoepidemiology of multiple sclerosis and environmental & infectious determinants of its clinical course

By

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration of originality

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania, nor any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief, no material previously published or written by another person except where due acknowledgement is made in the text of the thesis

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Statement of Co-authorship

This thesis includes papers for which Steve Simpson, Jr. (SSJ) is not sole author. SSJ took the lead in this research, developing and implementing the analyses included herein under the supervision of Leigh Blizzard (LB), writing manuscripts, and in the case of the meta-analysis of multiple sclerosis prevalence, designing and implementing the research project. In this process, however, he was assisted by co-authors to varying extent. Following then, the contributions of each co-author are detailed for each respective project.

1. The paper reported in Chapter 2:

Simpson, Jr. SL, Pittas F, van der Mei I, Blizzard L, Ponsonby A-L, Taylor B. "Trends in the epidemiology of multiple sclerosis in Greater Hobart, Tasmania: 1951 to 2009." *Journal of Neurology, Neurosurgery & Psychiatry*. Feb 2011; 82(2): 180-187.

- SSJ contributed to the data collection for the 2009 prevalence data along with Bruce Taylor (BT), management of the 2009 prevalence data and consolidation with the 2001 prevalence data, calculation of the 2001-2009 incidence and mortality rates; along with LB, statistical analysis of temporal trends in prevalence, incidence and mortality was done by SSJ under supervision by LB. SSJ composed drafts of the manuscript and coordinated revision.
- Fotini Pittas (FP) was involved in the development and acquisition of funding for both the 2001 and 2009 prevalence studies along with BT and Ingrid van der Mei (IvM); FP contributed to the data collection for the 2001 prevalence data along with BT; FP was involved in the conception of some of the analyses used in the study and contributed to the critical revision of the manuscript.
- LB provided guidance and supervision for all statistical analyses undertaken in this study, and was involved in the initial drafting and critical revision of the manuscript.

- IvM was involved in the development and acquisition of funding for both the 2001 and 2009 prevalence studies along with BT and FP; IvM was involved in the initial drafting and critical revision of the manuscript.
- Anne-Louise Ponsonby (A-LP) was involved in the development and acquisition of funding for the 2001 prevalence study along with BT, IvM and FP, and contributed to the critical revision of the manuscript.
- BT was involved in the development acquisition of funding for both the 2001 and 2009 prevalence studies along with IvM and FP. BT was involved in data collection for both the 2001 and 2009 prevalence studies. BT was involved in the initial drafting and critical revision of the manuscript.

2. The paper reported in Chapter 3:

Simpson, Jr. SL, Blizzard, L, Otahal P, van der Mei I, Taylor B. "Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis." *Journal of Neurology, Neurosurgery & Psychiatry*. 82(10); 1132-1141.

- SSJ conceived the project and collected all prevalence data required, from published manuscripts, conference proceedings and/or direct correspondence with study authors. In concert with and under guidance of LB, Petr Otahal (PO) and BT, SSJ developed and implemented all statistical analyses. SSJ composed the drafts of the manuscript and coordinated revision. SSJ consolidated the data and composed the table in Appendix 4A. SSJ consolidated the data and composed the initial draft and critical revision of Appendix 4B. SSJ composed the initial draft and critical revision of Appendix 4C. SSJ consolidated the data and composed the table in Appendix 4D.
- LB provided guidance and supervision for all statistical analyses undertaken in this study, and was involved in the initial drafting and critical revision of the manuscript.

- PO worked in concert with SSJ, LB and BT to develop statistical analyses undertaken in this study, and was involved in critical revision of the manuscript.
- IvM was involved in the initial drafting and critical revision of the manuscript.
- BT worked in concert with SSJ, PO, and PO to develop analyses undertaken in this study, and was involved in the initial drafting and critical revision of the manuscript.

3. The paper reported in Chapter 4:

Simpson, Jr. SL, Greenhill K, van der Mei I, Stankovich J, Charlesworth J, Taylor B. "The varied mechanisms of vitamin D in the onset and clinical course of MS: potential roles in modulating other aetiologic pathways." *Current Medical Literature – Neurology*. 27(1) 1-14.

- SSJ undertook the literature review for the background and immunological actions of vitamin D and its metabolites, epidemiology of personal UV exposure, vitamin D intake and circulating levels of vitamin D and their relationship with multiple sclerosis risk and clinical course, and the role of vitamin D in manifesting or modulating other causal pathways in multiple sclerosis risk and clinical course. SSJ composed the initial draft of these sections and coordinated critical revision of the manuscript.
- Kate Greenhill (KG) undertook the literature review for the background and intracellular and genetic actions of 1,25-dihydroxyvitamin D. KG composed the initial draft of these sections and contributed to the critical revision of the manuscript.
- IvM contributed to the critical revision of the manuscript.
- Jim Stankovich (JS) provided guidance for KG and contributed to the critical revision of the manuscript.
- Jac Charlesworth (JC) provided guidance for KG and contributed to the critical revision of the manuscript.
- BT contributed to the critical revision of the manuscript.

4. The paper reported in Chapter 5:

Simpson Jr. SL, Taylor B, Blizzard L, Ponsonby A-L, Pittas F, Tremlett H, Dwyer T, Gies P, van der Mei I. "Higher 25-hydroxyvitamin D is associated with lower relapse risk in MS." *Annals of Neurology*. Aug 2010; 68(2): 193-203.

- SSJ was involved in the development and implementation of statistical analyses undertaken, under supervision by LB. SSJ composed the drafts of the manuscript and coordinated revision.
- BT was involved in the development and acquisition of funding for the MS Longitudinal Study from which the data for this analysis was drawn, along with A-LP, FP, Terence Dwyer (TD), Peter Gies (PG), and IvM; BT was involved in the data collection for the MS Longitudinal Study, along with FP, PG, and IvM. BT was involved in the initial drafting and critical revision of the manuscript.
- LB provided guidance and supervision for all statistical analyses undertaken in this study, and was involved in the initial drafting and critical revision of the manuscript.
- A-LP was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, FP, TD, PG, and IvM; A-LP contributed to the critical revision of the manuscript.
- FP was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, TD, PG, and IvM. FP was involved in the data collection for the MS Longitudinal Study, along with BT, PG, and IvM. FP contributed to the critical revision of the manuscript.
- Helen Tremlett (HT) was involved in the conception and implementation of the analyses used and contributed to the critical revision of the manuscript.

- TD was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, FP, PG, and IvM. TD contributed to the critical revision of the manuscript.
- PG was involved in the data collection for the MS Longitudinal Study, specifically the acquisition and reading of the polysulphone badge data. PG contributed to the critical revision of the manuscript.
- IvM was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, FP, and TD. IvM was involved in the data collection for the MS Longitudinal Study, along with BT, FP and PG. IvM was involved in the conception of the analyses used. IvM was involved in the initial drafting and critical revision of the manuscript.

5. The paper reported in Chapter 6

Simpson Jr. SL & Stewart N, van der Mei I, Eyles D, Ko P, Ponsonby A-L, Pittas F, Blizzard L, Dwyer T, Taylor B. "Interferon-β is associated with higher serum 25-hydroxyvitamin D and both interact to modulate relapse risk in multiple sclerosis." *Neurology*. In-press (Accepted 15 November 2011).

- SSJ was involved in the development and implementation of statistical analyses undertaken, under supervision by LB. SSJ composed the drafts of the manuscript and coordinated revision.
- NS initiated project and was involved in initial drafting and critical revision of the manuscript.
- IvM was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, FP, and TD. IvM was involved in the data collection for the MS Longitudinal Study, along with BT and FP. IvM was involved in the critical revision of the manuscript.

- Darryl Eyles (DE) was involved in measurement of 1,25-dihydroxyvitamin D and was involved in critical revision of the manuscript.
- Pauline Ko (PO) was involved in measurement of 1,25-dihydroxyvitamin D and was involved in critical revision of the manuscript.
- A-LP was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, FP, TD, and IvM; A-LP contributed to the critical revision of the manuscript.
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- LB provided guidance and supervision for all statistical analyses undertaken in this study, and was involved in the initial drafting and critical revision of the manuscript.
- TD was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, FP, and IvM. TD contributed to the critical revision of the manuscript.
- BT was involved in the development and acquisition of funding for the MS Longitudinal Study from which the data for this analysis was drawn, along with A-LP, FP, TD, and IvM; BT was involved in the data collection for the MS Longitudinal Study, along with FP and IvM. BT was involved in the initial drafting and critical revision of the manuscript.

6. The paper reported in Chapter 7:

Simpson, Jr. SL, Taylor B, Dwyer D, Taylor J, Blizzard L, Ponsonby A-L, Pittas F, Dwyer T, van der Mei, I. "Anti-HHV-6 IgG titers are significantly predictive of relapse risk in multiple sclerosis." *Multiple Sclerosis.* **doi: 10.1177/1352458511428081.**

- SSJ was involved in the development and implementation of statistical analyses undertaken, under supervision by LB. SSJ composed the drafts of the manuscript and coordinated revision.
- BT was involved in the development and acquisition of funding for the MS Longitudinal Study from which the data for this analysis was drawn, along with A-LP, FP, TD, and IvM; BT was involved in the data collection for the MS Longitudinal Study, along with Dominic Dwyer (DD), Janette Taylor (JT), FP and IvM. BT was involved in the initial drafting and critical revision of the manuscript.
- DD was involved in the data collection for the MS Longitudinal Study, specifically the measurement of anti-human herpesvirus IgG titres, along with JT. DD was involved in the critical revision of the manuscript.
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- LB provided guidance and supervision for all statistical analyses undertaken in this study, and was involved in the initial drafting and critical revision of the manuscript.
- A-LP was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, FP, TD, and IvM; A-LP contributed to the critical revision of the manuscript.
- FP was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, TD, and IvM. FP was involved in the data collection for the MS Longitudinal Study, along with BT, DD, JT, and IvM. FP contributed to the critical revision of the manuscript.

- TD was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, FP, and IvM. TD contributed to the critical revision of the manuscript.
- IvM was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, FP, and TD. IvM was involved in the data collection for the MS Longitudinal Study, along with BT, DD, JT and FP. IvM was involved in the conception of the analyses used. IvM was involved in the initial drafting and critical revision of the manuscript.

7. The paper reported in Chapter 8:

Simpson, Jr. SL, Taylor B, Dwyer D, Taylor J, Blizzard L, Ponsonby A-L, Pittas F, Dwyer T, van der Mei, I. "Serological reactivation of human herpesvirus 6 is not associated with clinical outcomes in multiple sclerosis." (unsubmitted manuscript)

- SSJ was involved in the development and implementation of statistical analyses undertaken, under supervision by LB. SSJ composed the drafts of the manuscript and coordinated revision.
- BT was involved in the development and acquisition of funding for the MS Longitudinal Study from which the data for this analysis was drawn, along with A-LP, FP, TD, and IvM; BT was involved in the data collection for the MS Longitudinal Study, along with Dominic Dwyer (DD), Janette Taylor (JT), FP and IvM. BT was involved in the initial drafting and critical revision of the manuscript.
- DD was involved in the data collection for the MS Longitudinal Study, specifically the measurement of anti-human herpesvirus IgG titres, along with JT. DD was involved in the critical revision of the manuscript.

- JT was involved in the data collection for the MS Longitudinal Study, specifically the measurement of anti-human herpesvirus IgG titres, along with DD. JT was involved in the critical revision of the manuscript.
- LB provided guidance and supervision for all statistical analyses undertaken in this study, and was involved in the initial drafting and critical revision of the manuscript.
- A-LP was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, FP, TD, and IvM; A-LP contributed to the critical revision of the manuscript.
- FP was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, TD, and IvM. FP was involved in the data collection for the MS Longitudinal Study, along with BT, DD, JT, and IvM. FP contributed to the critical revision of the manuscript.
- TD was involved in the development and acquisition of funding for the MS Longitudinal Study, along with BT, A-LP, FP, and IvM. TD contributed to the critical revision of the manuscript.
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Lastly, I would offer my thanks to the participants of the MS Longitudinal Study and the MS Prevalence & Genetics Study, as well as the innumerable participants in the studies included in my meta-analysis. The work we do would not exist were it not for their kind participation and giving of their time, so at the very least I hope that the work of my thesis in some way is of help to our participants, and all persons.

Abstract

Multiple sclerosis (MS) is a chronic, demyelinating condition of the central nervous system, manifesting in alteration or loss of motor, sensory and cognitive function. The causes of MS are unclear but include genetic and environmental factors. This thesis presents several epidemiologic analyses, examining MS geoepidemiology, locally and globally, as well as evaluating key environmental and infectious determinants of clinical course.

The first analysis chapter examines MS epidemiology in the Greater Hobart region of Tasmania over the interval 1951 to 2009. This analysis found a significant increase in prevalence, this mediated by a significantly decreased mortality and increased longevity, as well as evidence of an increasing female/male sex ratio.

Next is a meta-analysis of MS prevalence and its association with latitude. This work, utilising the largest collection of MS prevalence studies, found a significant positive association between MS prevalence and latitude. This provides evidence in favour of the latitudinal gradient hypothesis and for environmental factors underlying the gradient, most particularly personal ultraviolet radiation (UVR) exposure and vitamin D.

The association between serum 25-hydroxyvitamin D (25(OH)D) and relapse was examined in a prospective cohort with clinically-definite MS followed for 2.3 years. This analysis found a significant inverse association between higher levels of 25(OH)D and subsequent hazard of relapse. This study provides key evidence that is needed to justify conducting randomised clinical trials of vitamin D supplementation in reducing relapse frequency in MS.

In this MS cohort, it was also found that persons on interferon-β (IFN-β) therapy had significantly higher 25(OH)D levels and that the association between personal sun exposure and 25(OH)D was stronger compared to those not on IFN-β. Importantly, the above association between 25(OH)D and relapse was only observed for those on IFN-β therapy.

Abstract

Last is an examination of the role of antibodies to Human Herpesvirus 6 (HHV-6) and Epstein-Barr virus (EBV) in MS clinical course. This analysis found a significant positive association between anti-HHV-6 IgG and relapse. This effect persisted on adjustment for the anti-EBV IgGs, indicating the effect was specific for HHV-6 antigen, or host antigen resembling it. There was no evidence of frequent serological HHV-6 reactivation, suggesting that the observed association between anti-HHV-6 IgG and relapse was not being mediated by serologically-detectable peripheral reactivation of HHV-6. No associations were observed between anti-HHV-6 and anti-EBV IgGs and progression in clinical disability.

This thesis presents a range of studies which add significantly to the literature on MS geoepidemiology, as well as the associations of environmental and infectious factors on MS clinical course. This work will be useful in the scientific community; both for hypothesis generation and providing strong evidence in support of existing hypotheses, and hopefully be of benefit to people with this debilitating disease.

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Papers directly arising from the work described in this thesis

Papers published

Chapter 2:

Simpson, Jr. SL, Pittas F, van der Mei I, Blizzard L, Ponsonby A-L, Taylor B. "Trends in the epidemiology of multiple sclerosis in Greater Hobart, Tasmania: 1951 to 2009." *Journal of Neurology, Neurosurgery & Psychiatry*. Feb 2011; 82(2): 180-187. [ERA 2010: A]

Chapter 3:

Simpson, Jr. SL, Blizzard, L, Otahal P, van der Mei I, Taylor B. "Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis." *Journal of Neurology, Neurosurgery & Psychiatry*. 82(10); 1132-1141. [ERA 2010: A]

Chapter 4:

Simpson, Jr. SL, Greenhill K, van der Mei I, Stankovich J, Charlesworth J, Taylor B. "The varied mechanisms of vitamin D in the onset and clinical course of MS: potential roles in modulating other aetiologic pathways." *Current Medical Literature – Neurology*. 2011; 27(1): 1-14. [ERA 2010: NR]

Chapter 5:

Simpson Jr. SL, Taylor B, Blizzard L, Ponsonby A-L, Pittas F, Tremlett H, Dwyer T, Gies P, van der Mei I. "Higher 25-hydroxyvitamin D is associated with lower relapse risk in MS." *Annals of Neurology*. Aug 2010; 68(2): 193-203. [ERA 2010: A*]

Chapter 6:

Simpson Jr. SL & Stewart N, van der Mei I, Eyles D, Ko P, Ponsonby A-L, Pittas F, Blizzard L, Dwyer T, Taylor B. "Interferon-β is associated with higher serum 25-hydroxyvitamin D and both interact to modulate relapse risk in multiple sclerosis." *Neurology*. In-press (Accepted 15 November 2011). [ERA 2010: A]

Chapter 7:

Simpson, Jr. SL, Taylor B, Dwyer D, Taylor J, Blizzard L, Ponsonby A-L, Pittas F, Dwyer T, van der Mei, I. "Anti-HHV-6 IgG titers are significantly predictive of relapse risk in multiple sclerosis." *Multiple Sclerosis.* **doi: 10.1177/1352458511428081.** [ERA 2010: A]

Other publications

van der Mei I, **Simpson, Jr. SL**, Stankovich J, Taylor B. "Individual and joint action of environmental factors and risk of MS", *Neurologic Clinics*. May 2011; 29; 233 – 255. [ERA 2010: NR]

van der Mei, I, **Simpson, Jr. SL**, Knippenberg S, Winzenberg T, Taylor BV. "The role of vitamin D in multiple sclerosis: implications for disease management" *Neurodegenerative Disease Management* (Invited review). 1(6); 523-36. [ERA 2010: NR]

Conference presentations arising from work in this thesis

Oral presentations

2009 "Increasing levels of vitamin D are associated with decreased hazard of relapse in multiple sclerosis", *2009 Australian and New Zealand Association of Neurologists Annual Scientific Meeting*, Christchurch, NZ, 17-21 May 2009.

"The epidemiology of multiple sclerosis in Greater Hobart, Tasmania: 1951-2009", *Multiple Sclerosis Research Association Progress in MS Research Scientific Conference*, Sydney, 14-17 Oct 2009.

2010 "Latitude is positively associated with the prevalence of multiple sclerosis: a definitive demonstration of the latitudinal gradient hypothesis using meta-analysis", *Australasian Epidemiological Association Annual Meeting*, Sydney, 29 Sept-1 Oct 2010.

"The role of human herpesvirus 6 in the clinical course of multiple sclerosis", *2010 Student Excellent in Research Conference,* Hobart 16-17 Nov 2010.

2011 "Anti-HHV IgG and clinical outcomes in multiple sclerosis", *2011 Australian and New Zealand Association of Neurologists Annual Scientific Meeting,* Hobart, 16-19 May 2011.

"Interferon-β is associated with higher 25-hydroxyvitamin D and both interact to modulate relapse risk in multiple sclerosis", *Multiple Sclerosis Research Association Progress in MS Research Conference*, Melbourne, 26-28 Oct 2011.

Poster presentations

2009 "The epidemiology of multiple sclerosis in Greater Hobart, Tasmania: 1951-2009", *2009 Australian and New Zealand Association of Neurologists Annual Scientific Meeting*, Christchurch, NZ, 17-21 May 2009.

"Increasing levels of vitamin D are associated with decreased hazard of relapse in multiple sclerosis", *Multiple Sclerosis Research Association Progress in MS Research Scientific Conference*, Sydney, 14-17 Oct 2009.

Awards received from the work described in this thesis

Young Investigator Award for best oral presentation at the *Multiple Sclerosis Research Association Progress in MS Research Conference*, Melbourne, 26-28 Oct 2011.

List of abbreviations

Abbreviation	Full term
µg	Micrograms
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
95% CI	95 percent Confidence Interval
ABS	Australian Bureau of Statistics
AHR	Adjusted Hazard Ratio
APC	Antigen Presenting Cell
BMI	Body Mass Index
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DIS	Dissemination in space
DIT	Dissemination in time
EBNA	Epstein-Barr Nuclear Antigen
EBV	Epstein-Barr Virus
EBV-EA	Epstein-Barr Virus Early Antigen
EDSS	Kurtzke Expanded Disability Severity Scale
EIA	Enzyme-linked Immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
HERV	Human Endogenous Retrovirus
HHV	Human Herpesvirus
HHV-6	Human Herpesvirus 6
HLA	Human Leukocyte Antigen
HLA-DRB1	Most prevalent beta-subunit for Class II Major Histocompatibility Complex
HR	Hazard Ratio
IFA	Immunofluorescence Assay
IFN-β	Interferon beta
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
IL	Interleukin
IQR	Interquartile Range
ISR	Incidence Sex Ratio
IU	International Units
Km	Kilometers
MET	Metabolic Equivalent of Task
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MSFC	Multiple Sclerosis Functional Composite
MSL	Multiple Sclerosis Longitudinal Study
MSSS	Multiple Sclerosis Severity Score
nmol/L	Nanomoles per Liter
NSW	New South Wales
PBMC	Peripheral Blood Monocyte
PCR	Polymerase Chain Reaction
PPMS	Primary-Progressive Multiple Sclerosis
PSR	Prevalence Sex Ratio
RCT	Randomised Controlled Trial

RRMS	Relapsing-Remitting Multiple Sclerosis
RTI	Respiratory Tract Infection
SD	Standard Deviation
SED	Standard Erythemal Dose
SPMS	Secondary-Progressive Multiple Sclerosis
TAS	Tasmania
T_h1	Helper T-lymphocyte class 1
T_h2	Helper T-lymphocyte class 2
T_h17	Helper T-lymphocyte class 17
T_{reg}	Regulatory T-lymphocyte
WA (Australia)	Western Australia
UK	United Kingdom of Great Britain and Northern Ireland
USA	United States of America
UVR	Ultraviolet radiation
VCA	Epstein-Barr Virus Viral Capsid Antigen
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VDRE	Vitamin D receptor element
vIL-10	viral interleukin 10

Chapter 1. Background on multiple sclerosis, its history, pathophysiology, diagnosis and treatment

1.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune condition of the central nervous system (CNS), manifesting in alterations in and/or loss of neurological function, including sensory, motor and cognitive. The causes of MS are complex, and the exact causes of the disease and drivers of its clinical course remain unclear. Keys to teasing out the causal mechanisms of the disease, however, may be found via epidemiological research, interpreting biological specimens, environmental exposures and behaviour to find associations with disease occurrence and clinical course.

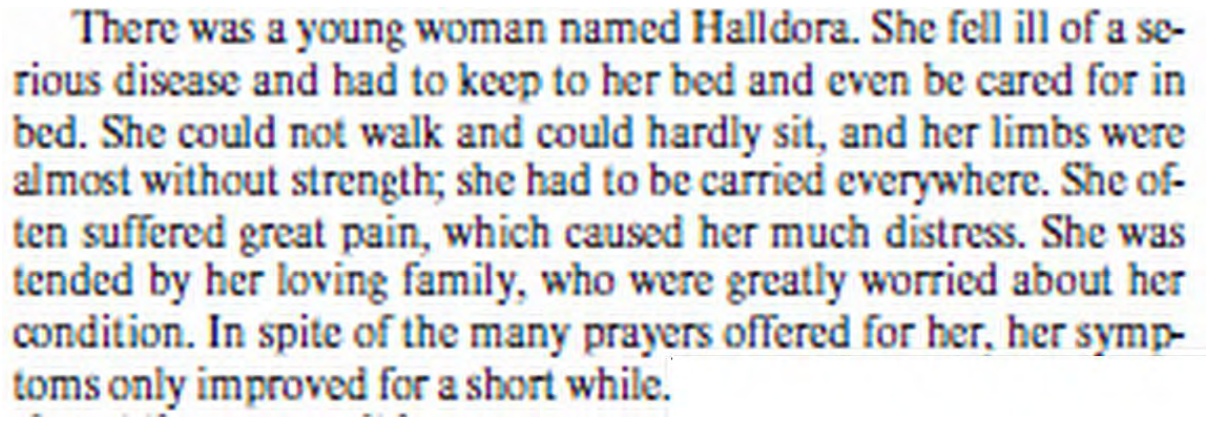
The research presented in this thesis is the result of such epidemiological research: the first two analyses examine the local and global distribution of MS occurrence, the interpretation of which may glean prediction of the temporal and spatial dynamics of disease; the subsequent analyses evaluate environmental and infectious determinants of clinical course, which help understand some of the underlying pathology and may be of use in development of diagnostic and treatment applications in MS.

While the work presented herein is epidemiological, with no pathological, immunological or biomolecular analyses having been undertaken, a brief discussion of these aspects, along with a summary of the history and current medical knowledge about MS, is necessary for context. In addition will be presented information specific to the subject areas discussed in analyses included in this thesis, including the epidemiology of MS in Greater Hobart and the distribution of MS globally, the role of vitamin D in MS onset and clinical course, and the role of human herpesviruses, particularly Epstein-Barr virus and human herpesvirus 6 in MS onset and clinical course.

1.2 The history of MS

Like many diseases of middle age, MS didn't reach notice until relatively recently in history. In contrast to the multivariate infectious ailments which felled most people before they reached their fourth decade of life, MS would have been sufficiently rare as to not rise to the attention of the amateur researchers and physicians of history and, where it was noticed likely attributed to any number of spiritual or otherwise incorporeal causes. Thus, though it has likely existed for as long as humans achieved sufficient population density as to allow co-evolution of the variety of infectious pathogens and commensals as exist today, it is only in the last several hundred years that it was recognised, and only in the mid-19th century that it was identified as a distinct condition.

The earliest record of an ailment suggestive of MS is recounted in the Saga of Bishop Thorlak, the patron saint of Iceland and Bishop of Iceland from 1178 to 1193, concerning a woman named Halldora(1). The story tells that the Bishop was able to cure Halldora, this cited as one of the miracles for which he was later beatified, though the description of Halldora's condition is perhaps more illuminating, for its similarity to progressive MS, sans the miraculous treatment:


There was a young woman named Halldora. She fell ill of a serious disease and had to keep to her bed and even be cared for in bed. She could not walk and could hardly sit, and her limbs were almost without strength; she had to be carried everywhere. She often suffered great pain, which caused her much distress. She was tended by her loving family, who were greatly worried about her condition. In spite of the many prayers offered for her, her symptoms only improved for a short while.

(1)

A case description which is often cited as possibly being MS is that of Lidwina of Scheidam, in the Netherlands, in the late 14th century(2). This devout woman, subsequently beatified, developed progressive disability in her teens before passing away at 37 years of age. More recent to this is the case

of Margaret Davis in the late 17th century, who had progressive 'lameness' for several years following a peripartum illness, progressing to her becoming bedridden and finally expiring. As described by Gough:

"Margaret the wife of Thomas Davis dyed on the 17th day of this instant, January, 1701. Shee tooke cold in childe-bearing, above twenty yeares beefore her death; shee was seized thereby with paine and lamenesse in her limbs, and made use of severall remedyes for cureing thereof, butt all proved ineffectual. At last, as shee was in an Apothecary's shop buying ointments and ingredients for fomentations my unde, Mr. Richard Baddely, an able chirurgeon, saw her and asked her how shee gott her lamenesse: shee sayd by takeing could in child-birth. Then says hee spare this charges and labour, for all the Doctors and Surgeons in England cannot cure it Thou mayest live long, butt thy strength will still decay. After this shee went to lytle more charges, onely when King James II. came his progresse to Shrewsbury, shee was admitted by the King's Doctors to goe to His Majesty for the Touch, which did her noe good. Shee was forced to use crootches almost 20 yeares agoe, and I thinke it is now 10 yeares since shee grew soe weake that shee was faine to bee carryed in persons' armes. About two years-and-an-halfe before her death, shee kept her bedde continually."

(3)

More recent still, and of great utility for its detail, is the case of Augustus d'Este who, following a loss of vision after the funeral of a friend in 1822, kept a diary of his illness for two decades. His initial loss of vision and subsequent recovery shortly thereafter, as well as a recurrence of the loss of vision alongside sensations of pain throughout his body, and later a total numbness and loss of function in his legs, are highly reminiscent of relapsing-remitting MS(2, 4). This latter episode is strikingly described by d'Este:

Now a new disease began to shew itself: every day I found *gradually* (by slow degrees) my strength leaving me: I could clearly perceive each succeeding day that I went up and down the staircase with greater difficulty. When I slapped myself sharply on the loins for the time it increased my strength.—A torpor or numbness and want of sensation became apparent about the end of the Backbone and the Perineum. At length about the 4th of December my strength of legs had quite left me, and twice in one day I fell down upon the floor in attempting to go to the closed stool without assistance; I was obliged to remain on the floor until my Servant came in and picked me up. I remained in this extreme state of weakness for about 21 days, during which period I fell down about 5 times (*never fainting*) from my legs not being strong enough to carry my body. I never once fainted or had any sort of fit:—*Debility, extreme* debility was the only cause of my falling.

(4)

The first mention of a case of MS in medical literature comes in 1824, by Charles Prosper Ollivier d'Angers in his "Traité des maladies de la moelle épinière", with the description of a patient in his 20s who developed weakness at 17, progressing to his requring a cane to walk(2, 5). The first illustrative depictions of MS pathology were done by Robert Carswell in 1822(2), published amidst a number of illustrations of neurological pathologies he drew for a medical atlas. The first (Figure 1.1), what Carswell attributed to "cartilaginous transformation of the spinal arachnoid of old persons", is strongly suggestive of demyelinated lesions throughout the spinal cord(6). The second (Figure 1.2), which Carswell called "a peculiar disease state of the chord and pons Varolii, accompanied with atrophy of the discoloured portions", is today regarded as a classical depiction of MS and is frequently cited as the first such illustration(6).

Figure 1.1. Depiction of spinal cord abnormality likely to be MS, by Carswell.

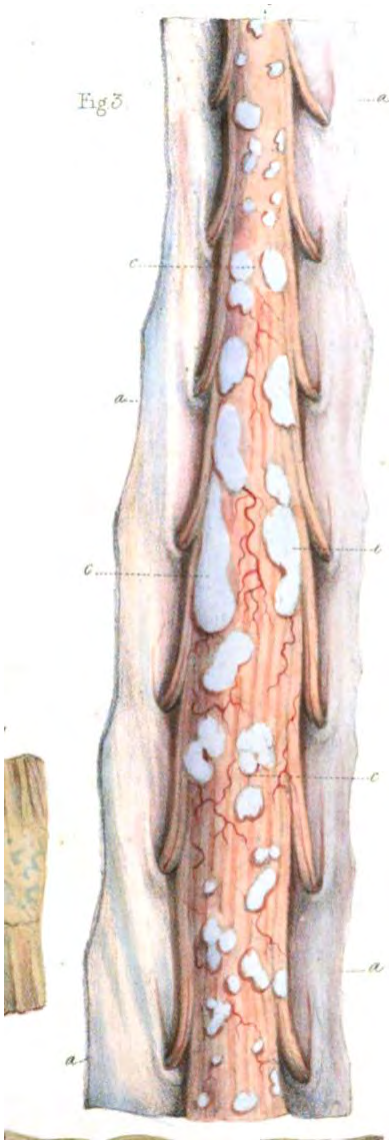
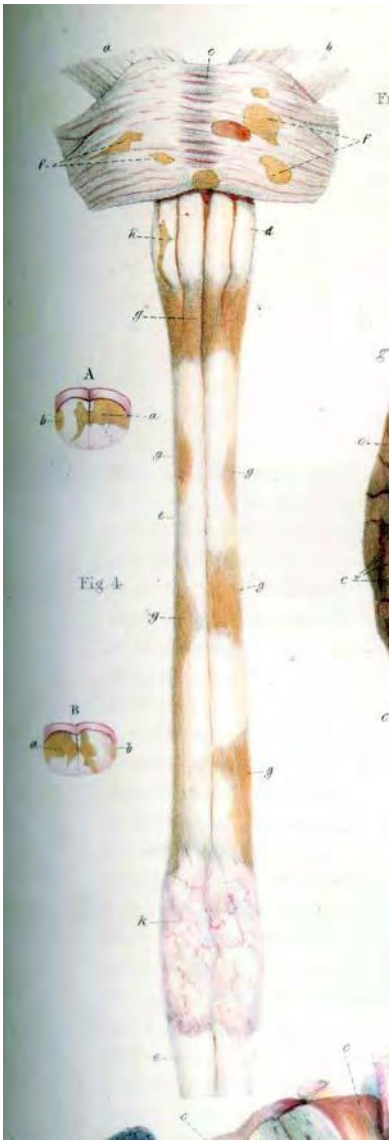


Figure 1.2. Depiction of spinal cord and pons abnormalities likely to be MS, by Carswell.



Figures reproduced from Carswell 1838(6)

In parallel with the work by Carswell, however, Jean Cruveilhier was developing a series of pathology lithographs (Figure 1.3, Figure 1.4). Indeed, it was Cruveilhier's work which was cited by Charcot in his later work (see below), though historical credit for the first illustrations of MS pathology goes to Carswell(2)

Figure 1.3. Lithograph of CNS abnormalities likely to be MS, by Cruveilhier

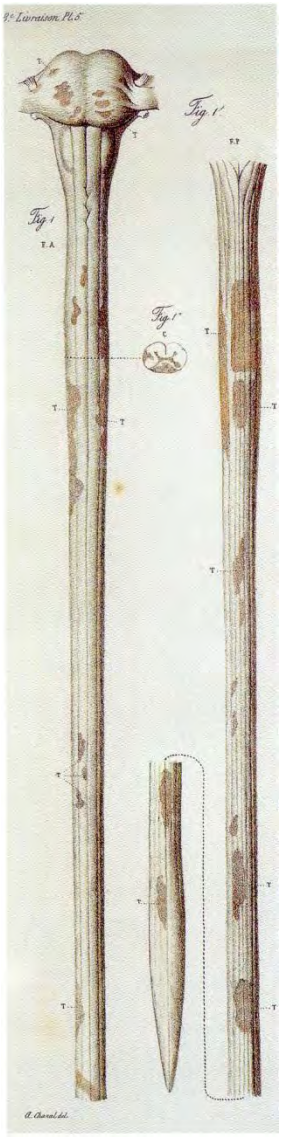
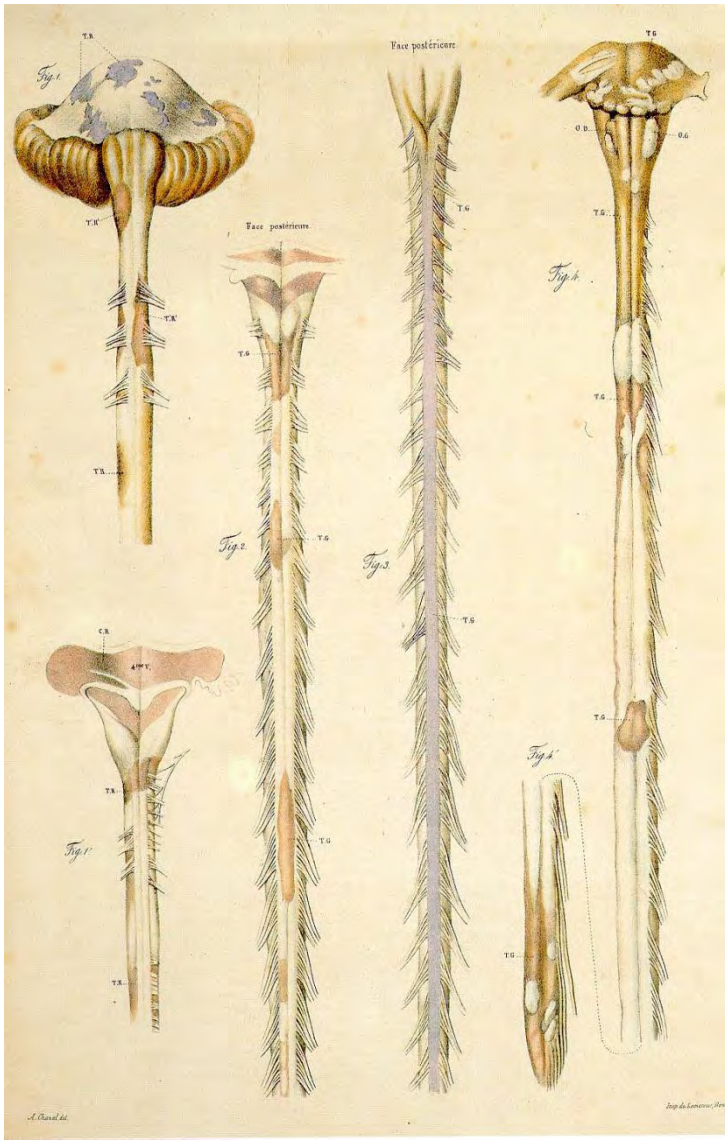


Figure 1.4. Lithograph of CNS abnormalities likely to be MS, by Cruveilhier



Figures reproduced from Cruveilhier 1835(7)

These historical instances of MS are retrospectively diagnosed today, but the first instances of disease being diagnosed as a discrete condition came in the mid-19th century. In much the same fashion as Carswell and Cruveilhier, characterisation of the disease of MS was come upon around the same time by Friedrich Theodor von Frerichs in Germany and Jean-Martin Charcot and Edmé Felix Alfred Vulpain in France, the former calling it "Hernsklerose", while Vulpain called it "la sclerose en plaque

disseminеé"(2). Von Frerichs noted the disease's relapsing-remitting course and that it could evolve to a progressive course over time, as well as the frequency of nystagmus as a symptom. Charcot contributed the eponymous "Charcot's Triad", namely nystagmus, intention tremor, and telegraphic speech, as key symptoms diagnostic of MS, though this was later recognised as not entirely specific to MS(2). It was ultimately to Charcot that historical credit would go, though it has been argued that von Frerichs is the more deserved. In parallel with these findings are those by Eduard Rindfleisch, who noted that inflammatory lesions in MS are frequently perivascular, as well as noting its typical onset in patients' early 20s, and was the first to note the predominance of MS in females relative to males(2).

1.3 The epidemiology of MS

These latter elements discovered by Rindfleisch regarding the distribution of MS in the population, however basic, are fundamental examples of epidemiology, and such information is critical to understanding how any disease works. For any disease, but particularly one so complex as MS, involving such a complex system as the central nervous system, epidemiology is abundantly useful in helping generate hypotheses, and targeting the biomolecular and pathology research which identifies the precise disease processes underway. Similarly basic but fundamental findings as those early studies of MS distribution include showing MS is more common in higher latitude (8, 9), suggesting that latitude and UV may play a role(10), that certain groups are more at risk for MS than others, suggesting a role for genetics(11), that migration affects MS risk(12), suggesting some interplay between genetics and environment on MS risk(13), that herpesvirus exposure may relate to MS risk(14-16), that smoking may relate to exacerbation(17), and that acute infection may relate to exacerbation(18). Such findings are critical to identifying possible factors involved in MS, as well as providing evidence against other not likely to be involved.

1.3.1 MS in Greater Hobart, Tasmania

Given the high frequencies of MS in Hobart – consistently the highest in Australia – this area has long been a point of interest for MS research. Indeed, the relationship between the biomedical research

community and the prevalent population of persons with MS in southern Tasmania has yielded years of fruitful research of global import. The first systematic evaluation of MS epidemiology in Hobart was done by McCall and colleagues(19) in 1961, the initial three-city study of MS in Hobart in Tasmania, Perth in Western Australia, and Newcastle in New South Wales. This study found that despite its small population and geographically peripheral location, Hobart had the highest prevalence and incidence of MS in the nation, double that of Newcastle and Perth. This difference could not be attributed to differences in diagnosis – all cases were diagnosed using the Allison & Millar criteria(20) - and case ascertainment, nor access to care or significant differences in population structure, either age or ethnicity. Thus this study was among the first strong demonstrations of the potent difference in MS frequency by latitude. The follow-up three-city study of Hobart, Perth and Newcastle by Hammond and colleagues(21) in 1981, found Hobart continued to have the highest frequencies in Australia. Moreover, in all three sites, the prevalence of MS had nearly doubled in the 20 years since the preceding study by McCall (Figure 1.5).

Figure 1.5. MS prevalence in Australia: Hobart, Perth and Newcastle, 1961 and 1981.

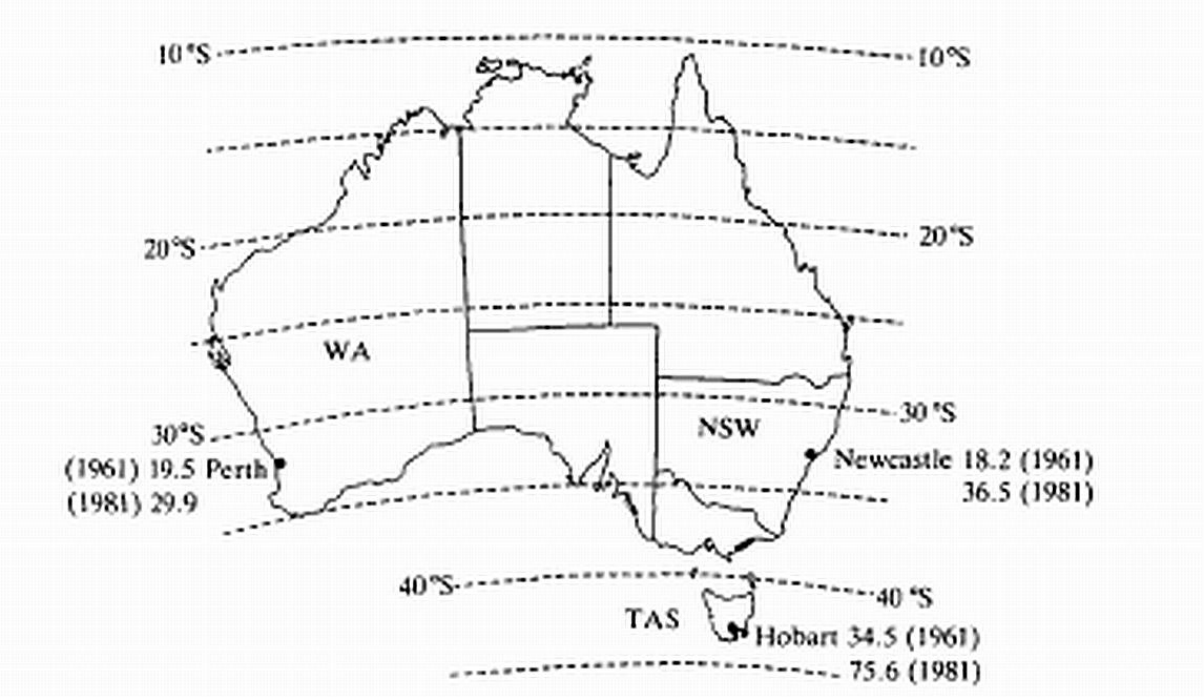


Figure reproduced from Hammond and colleagues(21)

Here again, the consistent methods used across each of the three sites, including diagnostic criteria – all cases diagnosed by the Rose criteria(22) – and relatively equal access to care and case ascertainment argues against ascription of the difference by site to methodological differences.

After these two studies, however, follow-up studies of MS epidemiology in Australia were relatively few. Hammond and colleagues undertook a study of Queensland(23) and McLeod evaluated the prevalence of MS in the states of New South Wales and South Australia(24), while Simmons evaluated the Australian Capital Territory(25). It was only in 2003, however, that Barnett and colleagues(26) evaluated one of the original three cities, assessing the prevalence in Newcastle in 1997. This study found a continued increase in the prevalence and incidence of MS as in the preceding two studies. No follow-up studies had yet been undertaken to evaluate the frequency of MS in Hobart, however. Thus

was undertaken a study of MS prevalence in 2001 and 2009, and incidence and mortality between 2001 and 2009, the results of which were published in 2011(27) and are reported in Chapter 2.

1.3.2 MS latitudinal gradient

The above-noted excess of MS cases in Hobart relative to the mainland of Australia had long been noted anecdotally by physicians and described generally by early researchers. Sutherland and colleagues(28), using mortality statistics in Australia and New Zealand, estimated the frequencies of MS in the populations above and below latitude 35°S, with those further south having nearly double the frequency of the northern locales. Similar work by Sutherland and colleagues(29) in the Australian state of Queensland found that the frequency of MS in the southern portion of the state (south of the Tropic of Capricorn, 23.5°) was nearly double that of the north; a follow-up study by Hammond and colleagues(23) in 1987 found this persisted. In parallel with work in Australia, studies of MS epidemiology in New Zealand have found a potent association between latitude and MS prevalence, despite the much shorter latitudinal range (33 vs. 12 degrees latitude). Skegg and colleagues(30) found the prevalence in the Southland/Otago region of the South Island to be nearly three-times that of the Waikato region of the North Island. Recently, a comprehensive study of MS prevalence in New Zealand by Taylor and colleagues(31) found this gradient persisted, with the Southland/Otago prevalence over double that of the Waikato region.

While a prototypical region for the study of MS geoepidemiology, given the largely standard medical infrastructure and access to care, and relative culture and ethnic homogeneity, Australasia is not the only region wherein a gradient in MS frequency has been noted. Indeed, anecdotal note of the gradient has been noted in the British Commonwealth nations of the mid-1900s(8), in Europe(32-36), North America(37-40) and Japan(41, 42). Kurtzke was the first to compile all the extant data from MS prevalence studies globally and evaluate their distribution by latitude. From this, Kurtzke(43) described bands of low, medium and high prevalence, increasing with latitude. A subsequent, larger study by

Kurtzke(44, 45) revised this gradient hypothesis slightly, given the much higher prevalence in northern North America relative to Scandinavia, allowing the gradient ranges to vary by longitude(46, 47). Subsequent reviews(48, 49) continued to support the gradient hypothesis, though with increasing note made of exceptions in Scandinavia and Mediterranean Europe. However, in a 1994 review of MS epidemiology in Europe, Rosati(50) argued that the association with latitude was an oversimplification, pointing to studies undertaken in Mediterranean Europe after 1980 which found high prevalence in a Kurtzke medium-prevalence zone(43-45). Rosati next undertook a 2001 descriptive review of MS prevalence globally(51, 52), arguing that the occurrence of aberrations from the gradient demonstrated the oversimplicity of the gradient hypothesis, instead proposing that much of the difference in prevalence distribution was due to variations in genetic susceptibility and study methodologies.

The first meta-analysis of MS geoepidemiology was done by Zivadinov and colleagues in 2003(53), combining data from 69 prevalence and 22 incidence studies between 1980 and 1998. Importantly, in addition to analysing crude values, Zivadinov age-standardised prevalence, finding a significant gradient in the crude analysis ($p<0.001$), though this was attenuated on age-standardisation ($p=0.01$). No association between latitude and incidence was found after age-standardisation however ($p=0.779$).

In 2008, Alonso and Hérnan undertook a meta-analysis of MS incidence, reviewing 28 studies with 38 incidence points between 1966 and 2007(54). The authors reported that, in contradistinction to the findings by Zivadinov(53), there was a significant association between incidence and latitude, though weakened after 1980. Recently, Koch-Henriksen and Sørensen(55) published findings from a meta-analysis of 226 MS prevalence and incidence studies, reporting "modest" associations between prevalence and latitude in Europe and North America ($p=0.018$); with incidence, the authors found no association for Western Europe ($p=0.99$) or North America ($p=0.93$). Surprisingly, in Australasia, an archetype of the latitudinal gradient(19, 21, 31), the authors found no association between latitude and prevalence ($p=0.17$), nor incidence after adjusting for study year ($p=0.18$).

As previous systematic reviews suffered from a number of methodological shortcomings, including incomprehensive inclusivity of prevalence studies, failure to age-standardise or weight prevalence appropriately, and failure to take into account relevant covariates, most particularly prevalence year, a meta-analysis which corrected these shortfalls was undertaken, the results of which were published in 2011(56) and are reported in Chapter 3.

1.4 Pathology of multiple sclerosis

1.4.1 Neurobiology

All cognition, sensation and motor function is comprised of and mediated by the functions of neurons, the specialised cells of the nervous system, both in the periphery and in the central nervous system. Though there are many different types of neurons for the varying functions of the nervous system, the basic structure of a neuron is composed of three major components: the dendrites, which receive signals from other neurons, an axon which deliver signals to other neurons or to muscle cells, and the soma, or cell body, which contains all the major cell components(57)(Figure 1.6). Transmission of signal along the neuron occurs by way of serial depolarisation due to opening of voltage-gated sodium and potassium channels, releasing sodium and potassium stored inside the cell and in so doing, eliminating the electric potential across the cell membrane formed from the sequestration of the ions inside the cell. The sudden release of these ions results in a loss of the electric potential manifests as an electric signal, called an action potential, which induces voltage-gated channels further along the cell to open, and propagating the signal down the length of the cell. Transmission of signal between neurons and their target cells is by way of neurotransmitters, compounds which are released into the short space between cells called the synapse, these neurotransmitters binding to receptors on the target cell and inducing depolarisation there by acting on ligand-gated ion channels, resulting either in further transmission if the cell is another neuron, or in contraction if the target cell is a muscle cell(57). Key to the optimal conduction of signal along the axon, which can be as long as a meter, is the myelin sheath which wraps around it, each segment of myelin sheath separated from one another by a gap, called the Nodes of

Ranvier. Ion channels are clustered at these nodes and during signal conduction, the electric potential 'jumps' from one node to the next, markedly expediting the speed with which the signal moves down the axon(57, 58).

Figure 1.6. Basic neuronal structure

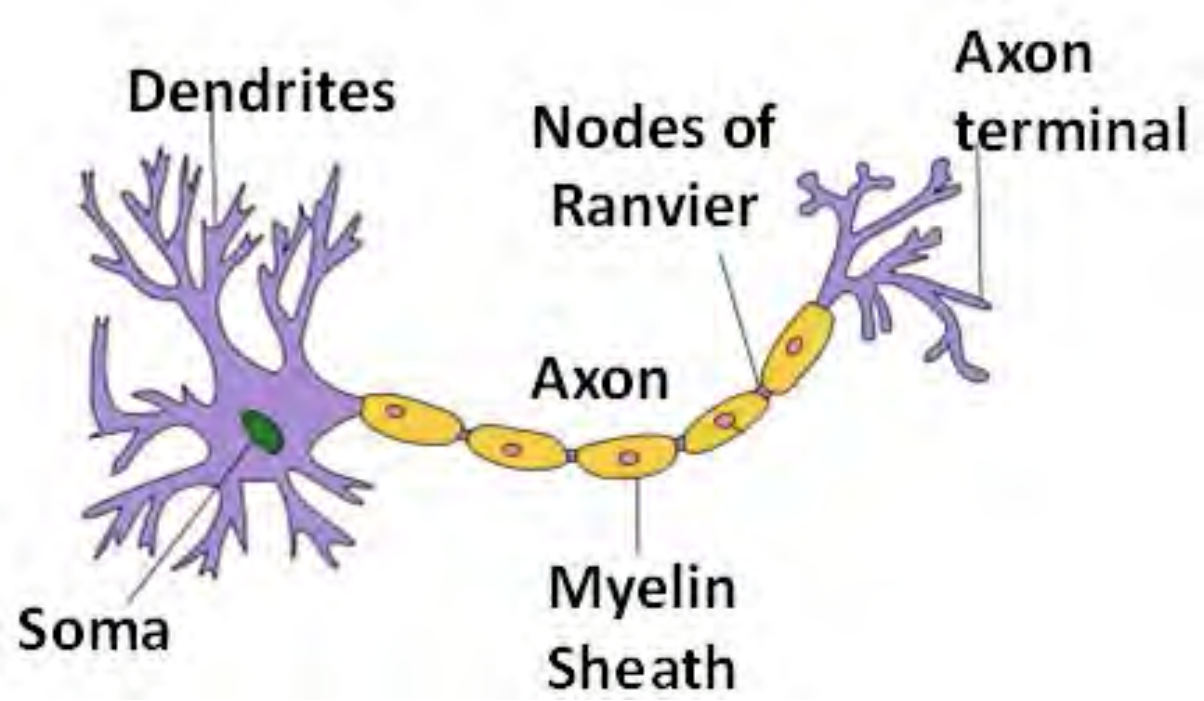


Figure reproduced from Cooper(58)

Within the CNS, amidst the neurons are a number of support cells, called glial cells, which include the microglia, astrocytes and oligodendrocytes. Microglia comprise roughly 20% of the glial cells of the CNS, and are analogous to the monocytes of the periphery (see section 1.4.2), and are the major sentinel cell of the CNS. Generally microglia are in a resting or ramified state, scanning the local environment for abnormalities but otherwise inactive. In response to inflammatory signals or other local stimuli, however, microglia become activated, adopting a more amoeboid structure, proliferating and producing inflammatory cytokines and phagocytising target materials(57). Another major glial cell in the CNS is the astrocyte, the most numerous glial cell in the CNS. This star-shaped cell is critical within the CNS, acting to maintain optimal environmental conditions within which neurons can survive,

regulating entry across the blood-brain-barrier, and actually involved in the physical structure of the brain(57). Astrocytes can also act in response to immunological stimuli, adopting a reactive state, proliferating, producing cytokines and, in response to physical damage to the brain or chronic inflammatory lesions, form an astroglial scar at the site(57). Of especial relevance for MS is the last type of glial cell, the oligodendrocyte, which produces and maintains the aforementioned myelin sheath which wrap around CNS neuronal axons(57)(Figure 1.7).

Figure 1.7. Neuroglial cells of the central nervous system

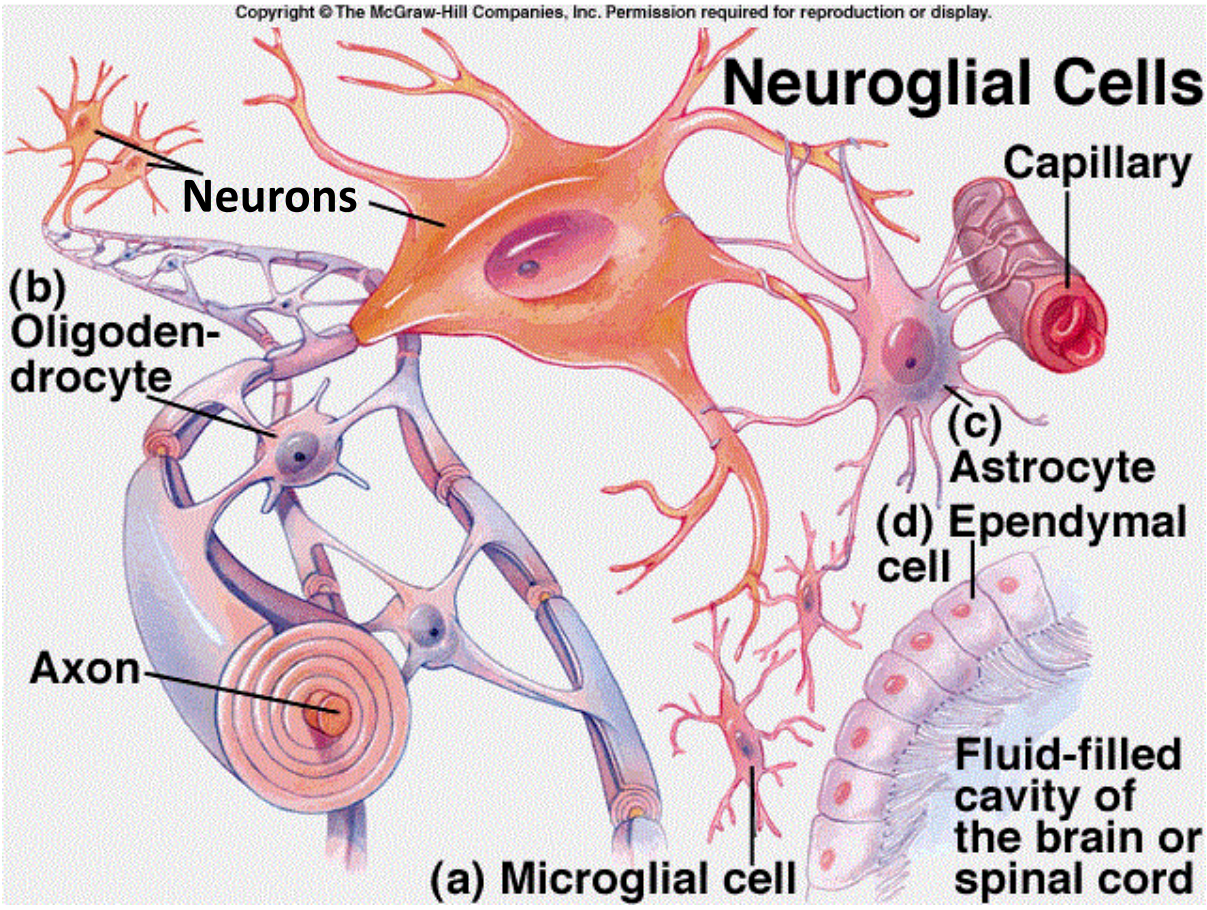


Figure reproduced from McGraw-Hill Companies, Inc.

Myelin is a critical component of the nervous system, comprising nearly 70% of the dry weight of the CNS in mammals(57). Myelinated axons are evident macroscopically as the "white matter" of the brain; "grey matter" is comprised of the neuronal cell bodies and non-myelinated axons and dendrites, and the glial cells(57). Myelin itself is largely made up of lipid (~75%), in line with its being an extension of

the oligodendrocyte membrane, but also contains a large amount of protein (~25%). Of the myelin protein, the two major proteins are proteolipid protein, found on the oligodendrocyte soma, and myelin basic protein, found on the sheath(57). Immunoreactivity to these proteins, particularly myelin basic protein, is suspected in the aetiology of MS autoimmunity(57).

1.4.2 Immunology

The immune system of the periphery is a complex system but may be reduced to two major components: innate and adaptive immunity, with adaptive immunity divided further into cell-mediated and humoral immunity(59, 60). Innate immunity is the most basic element of the immune system, and in addition to completely general components such as the skin and endothelium, which act to prevent microbial access to the body, also include non-specific but deliberately antimicrobial agents like macrophages and dendritic cells which attack and phagocytise microbes and other foreign material, and Toll-like receptors, which bind to various microbial components and induce inflammatory pathways(59, 60).

Macrophages and dendritic cells, upon phagocytising foreign matter, act within the adaptive immune system as antigen-presenting cells (APCs). These APCs break down the phagocytised material and various components are bound to its major histocompatibility complex (MHC) Type II receptor, which "presents" the antigen to $CD4^+$ T lymphocytes specific to that antigen. The T-cells bind to the MHC receptor on the APC with their T-cell receptor, and become activated, generating a specific immune response against that antigen. $CD4^+$ T-cells induce B-lymphocytes specific to the antigen to proliferate and produce immunoglobulin specific to that antigen, this the humoral immune response. Immunoglobulins, or antibodies, bind to the target antigen and can induce deposition of complement, which can lyse a target cell, or induce macrophages and dendritic cells to phagocytise the whole complex. APCs can also present antigen to $CD8^+$ T-lymphocytes, which attack and kill cells presenting

the target antigen on their MHC Class I surface proteins, this the cell-mediating immune response(59, 60).

$CD4^+$ T-cells may be divided still further by the types of cytokines they produce. These T-cell classes, Th1 and Th2, and more recently the Th17 cells, are defined by the cytokines they produce and reflect a particular immune response profile appropriate to the agent targeted and the stage of the response. Certain agents, such as bacteria and protozoa, are more efficiently targeted by a humoral response, and thus a Th2 response will favour induction of B-cell proliferation and immunoglobulin production, with production of IL-4, IL-10 and IL-13. Other targets, such as viral infections and tumour cells, require a cell-mediated immune response, since the infected or cancerous cells need be eliminated, so $CD8^+$ cells, natural killer cells and macrophages are induced, with production of cytokines like IL-1 and IFN-γ(59). The recently-discovered Th17 cells are a distinct inflammatory T-cell subset, being induced after chronic immune stimuli by combinations of IL-6 and TGF-β and producing IL-17(61). In addition to these inducers of immune response, the latter class of T-cells, called T-regulatory cells or Th3 cells, act to terminate the immune response, producing cytokines like TGF-β and IL-10(59).

Both MHCs and T-cell receptors are produced by random rearrangements of the corresponding genes, this yielding MHC and T-cells that can theoretically bind to any possible antigen. As these possible antigens include structures on host cells, the process by which T-cells mature includes a system to eliminate cells specific to "self" antigens, whereby developing T-cells in the thymus which bind to self-antigens are given signals which lead to anergy and apoptosis(59). However, due to various pathways, including failure to eliminate self-targeting T-cells, or molecular mimicry and epitope spreading, wherein an immune response against a legitimately foreign antigen initiates an immune response against a similar host antigen(59), autoimmune conditions exist against a number of self-antigens, manifesting in conditions like type 1 diabetes, rheumatoid arthritis and MS. As noted previously, the major self-

antigen generally considered relevant for MS is the myelin basic protein (MBP) found in the myelin sheath in the CNS, since an analogous disease to MS can be induced in animals called Experimental Autoimmune Encephalitis (EAE) following immunisation of animals with MBP(60).

1.4.3 Neuropathology

The major pathological feature of MS is the demyelinated lesion, this an area in the white matter which is nearly or completely devoid of myelin, with only bare axons or even axonal damage(62), though in later disease axons may be damaged, infiltration of peripheral immune cells, particularly T-cells and macrophages, and some element of astroglial scarring(63). Many lesions will be perivascular, this in keeping with the inflammatory processes whereby peripheral immune cells enter the CNS from circulation (63). Over the course of disease, an additional pathological element is brain atrophy, wherein the actual volume of the brain is reduced, this found especially in progressive courses of disease and correlating with permanent loss of function(63).

Due to processes not entirely clear, the immune system attacks the myelin surrounding the axons, leaving them bare and unable to conduct signal efficiently. Depending on the type of nerve affected and location within the brain and spinal cord, this can manifest in the alterations in cognition, sensation and motor function which define the condition(64). The major hypothesis for how damage occurs is that autoreactive T-cells specific for some component in myelin, possibly myelin basic protein, enter the CNS and attack the myelin and oligodendrocytes, produce inflammatory compounds and cytokines and generate an environment which is toxic for oligodendrocytes and neurons(60). The processes by which the autoimmune pathways leading to demyelination are initiated are unclear, however. The damage wrought in the course of disease are not constant: earlier in the course of disease, lesions are largely comprised of demyelinated axons but no loss of oligodendrocytes, allowing for rapid remyelination, whereas in more chronic lesions later in disease, demyelination occurs along with loss of

oligodendrocytes and formation of an astroglial scar, with demyelination and loss of function then being permanent(57, 65).

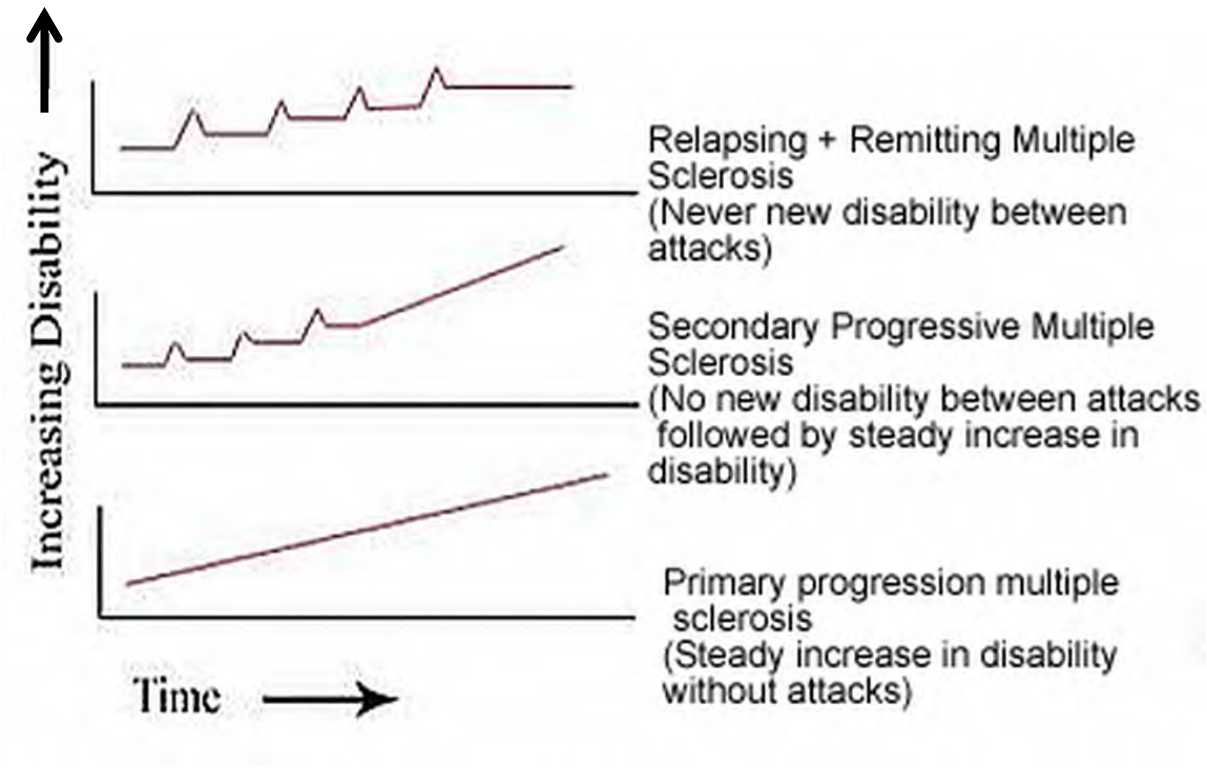
1.5 Clinical manifestation of MS

The clinical manifestation of MS is comprised of two principal components: relapse and progression. Relapse is a transient episode of increased disability, loss of or alterations in sensory, motor and/or cognitive function, not associated with change in temperature or fever, and lasting at least 24 hours(66). After some duration measured in days to weeks to months, symptoms will abate and the patient will return nearly to, but generally not all the way to, pre-relapse levels of function. Progression is a permanent and irreversible increase in disability and loss of function in some aspect of sensory, motor and/or cognitive function. Progression may occur in and of itself, as a chronic process of increasing disability and loss of function, or it may occur stepwise, due to the slight worsening after serial relapses.

1.5.1 MS courses

It is these two aspects of disease, relapse and progression, that define the types of MS within which a patient is classified, these types, or courses, of MS having a characteristic clinical profile and outlook, and which define the treatment regimens which can be utilised. The most common type of MS is relapsing-remitting MS (RRMS), comprising 80% of cases and defined by intermittent relapses with return to near pre-relapse levels of function (Figure 1.8). A subset of persons (~60%) with RRMS will after some amount of time convert to a progressive form of disease called secondary-progressive MS (SPMS), featuring no relapses but instead a steady progression to increased disability and loss of function. The other type of MS is distinct from RRMS and SPMS, featuring no relapses but instead a steady increase in disability and loss of function from disease onset, this called primary-progressive MS (PPMS)(63, 67). These disease types are not uniformly distributed in the population: females are more predisposed to a relapsing-remitting course, while males are more prone to the progressive types of disease(67).

Figure 1.8. Types of MS.



Figured modified from http://www.dwp.gov.uk

1.5.2 Relapse

While relapse is inherently an idiosyncratic phenomenon, with different symptoms from person to person, the overall event must abide within a standard definition, to distinguish it from the pseudorelapses which can occur due to fever or transient environmental heat exposure. Thus, the standard definition for relapse is derived from the 2001 McDonald Criteria for MS:

> An "attack" (exacerbation, relapse) refers to an episode of neurological disturbance of the kind seen in MS, when clinicopathological studies have established that the causative lesions are inflammatory and demyelinating in nature…an attack, defined either by subjective report or by objective observation, should last for at least 24 hours. This assumes that there is expert clinical assessment that the event is not a pseudoattack, such as might be caused by a change in core body temperature or infection."(66)

1.5.3 Progression

For progression, while a similarly idiosyncratic and complex phenomenon as relapse, there are several standard scales used to assess patients for their level of disability.

This was not always the case. The 1955 Disability Status Scale (DSS), by Kurtzke, was the first systematic scale for measuring neurological dysfunction and disability(68, 69). Prior to the DSS, neurological assessments were a subjective and fairly non-systematic affair, with measures of dysfunction in one system not necessarily proportionate to similar scores in another. This scale, with 1-unit increases ranging from 0 (normal) to 10 (death due to MS), assigned scores to individual subsystems and provided a systematic system for the measure of neurological dysfunction, allowing a uniform gradation of disability across a range of symptoms, marking a revolution in neurology(68, 69).

The most common scale used today was also proposed by Kurtzke in 1983, his Expanded Disability Status Scale (EDSS)(70). EDSS was a revision of the original DSS, expanding each 'step' of disability by 0.5, so as to provide a greater fineness of disability measure. EDSS allows neurologists to assign a functional system score to each of eight functional systems within neurological function: pyramidal, cerebellar, brainstem, sensory, bowel/bladder, visual, cerebral, and other, with a total score ranging from 0 (Normal) through to 10 (Death due to MS).

Derived from the EDSS is a scale which attempts to take into account duration of disease, the Multiple Sclerosis Severity Scale (MSSS)(71), which took a cohort of 9892 persons with clinically-definite MS of varying disability as measured by EDSS and varying durations of disease. This scale too ranges from 0 (Normal) to 10 (Death due to MS); however using an algorithm incorporating the disability and disease duration, the MSSS gives a higher score for equivalent levels of disability inversely proportionate to the duration of disease. Thus, a person with greater disability relatively early after diagnosis would have a higher MSSS than a person with similar disability that has accrued over many years of disease.

The Scripps Neurological Rating Scale was developed by the Scripps Clinic and includes 22 parameters for motor, sensory and cognitive function. Points are allocated proportionate to function (excepting bladder/bowel/sexual function which is inversely proportionate to function). These subscores are summated for a total Scripps score, ranging from 100 (Normal) to -10 (Maximal impairment)(72).

The Multiple Sclerosis Functional Composite (MSFC) is a discrete scale for measuring disability, making use of several measures of neurological function, including a timed 25 meter walk to measure balance and leg function/ambulation, a 9-hole peg test to measure coordination and arm/hand function, a 3-minute and 2-minute Paced Auditory Serial Addition Test (PASAT) to measure cognitive function, and a visual acuity test to measure visual function(73). Each test (excluding the visual acuity test) is done twice and the scores averaged and the mean used to calculate a Z-score for each section. The combined Z-scores from each of these sub areas are averaged to a continuous MSFC score.

1.5.4 Fatigue

An added dimension of MS that has long been recognised by patients, but only relatively recently been appreciated by physicians, is that of fatigue. In many ways intertwined with relapse and progression but also distinct, fatigue is frequently cited by patients as the major symptom which is most troubling and has the greatest effect on their quality of life(74).

Unlike the fairly objective assessment of 'normal' neurological function, fatigue is more subjective, incorporating level of disability, emotional state, and individual tolerance. Due to this, a variety of measures have been developed to measure fatigue(75-83). Each of the scales is as individual as the phenomenon under study, with relatively little overlap between scores(80). No one score has yet proved absolutely diagnostic in the same fashion as those of disability, and work is as yet in progress to develop a standard measure of this ubiquitous clinical feature.

1.6 MS diagnosis

1.6.1 Dissemination in space and time

Going back to the time of Charcot, when neurologists were attempting to classify the newly-identified condition to be known as MS, the major characteristics was that clinical symptoms, and later neuroimaging-determined lesions, be demonstrative of multiple lesions in space and in time. Dissemination in space (DIS) is needed since it identifies the condition as being that of MS, which is typically a pan-CNS affliction, in contrast to some other, more localised pathologies: "Evidence of multiple lesions…traditionally constitutes one of the corner-stones upon which the diagnosis of MS is built."(84) Dissemination in time (DIT) is needed so as to identify the condition as being distinct from a single event, a clinically-isolated syndrome: "The manner in which early symptoms tend to clear, partially or completely, only to return on one or more occasions has been recognized since the time of Charcot as a unique feature of the disease and one of major importance in diagnosis."(84) Historically, neurologists were obliged to rely on clinical markers of neurological abnormality in more than one area to prove DIS, while multiple episodes, either presenting at clinic or one clinic presentation and a verifiable history of separate episodes to prove DIS. More recently, the advent of paraclinical evidence, most particularly neuroimaging, has made diagnosis easier, both for the practitioner and the patient; however, fundamentally, modern diagnosis still relies on proof of DIS and DIT.

1.6.2 Paraclinical evidence

In attempting to prove the diagnosis of MS, clinical presentation has been and remains the bedrock of diagnosis. However, over time various paraclinical methods have been developed to give physicians an impression of neurological dysfunction which may not be clinically evident.

Among the first such evidence was alterations in the protein profile of cerebrospinal fluid (CSF). The early colloidal gold and mastix curves allowed a basic assessment of the protein density of CSF, with an 'abnormal' profile being utilised in some of the early criteria as supporting evidence for MS(85, 86). Later, more refined practices using gel electrophoresis allowed for the precise identification of bands

corresponding to immunoglobulins(87). These oligoclonal bands, polyspecific antibodies at abnormally high levels in the CSF, came to be a key component of paraclinical supporting evidence for MS diagnosis(88, 89).

Evoked potentials were utilised in various criteria of the mid to late 1900s, most particularly the McDonald-Halliday Criteria(90). Visual and audio evoked potentials featured the monitoring and measure of electrophysiological responses following visual and auditory stimuli, with aberrant response profiles being diagnostic for neurological dysfunction(91).

Of course the most prominent and frequently used paraclinical evidence today is that of magnetic resonance imaging (MRI). Indeed, to some extent MRI is encroaching upon the primacy of clinical evidence as a tool for diagnosis, with recent criteria relying almost entirely upon MRI evidence in their requirements for diagnosis(66, 92-96). MRI is a widely-used practice throughout medicine, allowing physicians previously unimaginable ability to assess internal pathologies and shape their diagnoses and treatments accordingly. In no other area perhaps but central nervous system medicine has MRI so revolutionised practice, allowing the in-vivo study and investigation of this heretofore unseen area of live human physiology. Making use of a targeted magnetic field to align hydrogen atoms in water molecules along a common axis and measuring their return to baseline state, an image of the internal structure of the target area can be realised(97). For MS, lesions viewed using T2-weighted MRI will appear bright white against the grey background, reflecting water molecules in the lesion, rather than the hydrophobic lipid myelin. More recently, the use of T1-weighted MRI with enhancing contrast agents, most particularly gadolinium which can only gain entrance where openings for vascular flow across the blood-brain-barrier exist, has allowed the assessment of the age of a given lesion, with a more recently-occurring lesion enhancing from gadolinium, while an older lesion without vascular access will not enhance(98). It is this difference in enhancement with lesion age that is allowing recent

criteria (92, 94-96) the long-sought ability to make a diagnosis from a single MRI scan, rather than serial scans or clinical history as required previously(66, 93).

1.6.3 Diagnostic criteria

Prior to systematic criteria, the neurologist was, even more than today, the sole arbiter in diagnosing MS, and diagnostic categorization was essentially divided between "typical" cases presentation and otherwise a-typical but probable or possible MS. In this early period, many of the same diagnostic tools used today, including clinical determination of DIS and DIT(99-102) and some use of laboratory methods such as oligoclonal bands in CSF and evoked potentials were used in some of the early prevalence studies(85, 86, 103-106). These earlier criteria are described in more detail in Appendix 3B. Here follows a brief description of the recent criteria, including the 2001 McDonald Criteria and its subsequent revisions.

1.6.3.1 2001 McDonald Criteria

The 1983 Poser criteria(107) were more or less the acknowledged diagnostic criteria in epidemiologic studies for the latter decades of the 20th century, until the development of the McDonald criteria in 2001(Figure 1.9). These criteria aimed to address some of the shortcomings in preceding criteria, and make greater use of neuroimaging as a diagnostic tool: whereas previously MRI had been only supporting evidence, the McDonald Criteria included them on the same level with clinical history and presentation, allowing a diagnosis to be made from a single MRI alongside appropriate clinical history, rather than requiring serial assessments.

Figure 1.9. 2001 McDonald criteria

Table 3. Diagnostic Criteria

Clinical Presentation	Additional Data Needed for MS Diagnosis
Two or more attacks; objective clinical evidence of 2 or more lesions	None[a]
Two or more attacks; objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by MRI[b] *or* Two or more MRI-detected lesions consistent with MS plus positive CSF[c] *or* Await further clinical attack implicating a different site
One attack; objective clinical evidence of 2 or more lesions	Dissemination in time, demonstrated by MRI[d] *or* Second clinical attack
One attack; objective clinical evidence of 1 lesion (monosymptomatic presentation; clinically isolated syndrome)	Dissemination in space, demonstrated by MRI[b] *or* Two or more MRI-detected lesions consistent with MS plus positive CSF[c] *and* Dissemination in time, demonstrated by MRI[d] *or* Second clinical attack
Insidious neurological progression suggestive of MS	Positive CSF[c] *and* Dissemination in space, demonstrated by 1) Nine or more T2 lesions in brain *or* 2) 2 or more lesions in spinal cord, *or* 3) 4–8 brain plus 1 spinal cord lesion *or* abnormal VEP[e] associated with 4–8 brain lesions, or with fewer than 4 brain lesions plus 1 spinal cord lesion demonstrated by MRI *and* Dissemination in time, demonstrated by MRI[d] *or* Continued progression for 1 year

If criteria indicated are fulfilled, the diagnosis is multiple sclerosis (MS); if the criteria are not completely met, the diagnosis is "possible MS"; if the criteria are fully explored and not met, the diagnosis is "not MS."

[a]No additional tests are required; however, if tests [magnetic resonance imaging (MRI), cerebral spinal fluid (CSF)] are undertaken and are *negative*, extreme caution should be taken before making a diagnosis of MS. Alternative diagnoses must be considered. There must be no better explanation for the clinical picture.
[b]MRI demonstration of space dissemination must fulfill the criteria derived from Barkhof et al[6] and Tintoré et al[7] (see Table 1).
[c]Positive CSF determined by oligoclonal bands detected by established methods (preferably isoelectric focusing) different from any such bands in serum or by a raised IgG index.[14,15]
[d]MRI demonstration of time dissemination must fulfill the criteria listed in Table 2.
[e]Abnormal visual evoked potential of the type seen in MS (delay with a well-preserved wave form).[16]

Reproduced from McDonald and colleagues(66).

1.6.3.2 2005 McDonald Criteria: Revision of the 2001 McDonald Criteria

Unlike previous criteria, where the previous criteria were entirely supplanted in favor of a new set, the 2005 Revision to the McDonald Criteria in 2005 was less a coup and more an adjustment. Indeed, rather than being the Polman Criteria, these criteria are known as the 2005 Revision of the McDonald Criteria. This revision took into account various issues and shortcomings(108-112) concerning the

2001 McDonald criteria and attempted to update and adapt to fix them, most particularly relaxing requirements for demonstration of DIT (Figure 1.10).

Figure 1.10. 2005 Revision to the McDonald Criteria

Table 4. The 2005 Revisions to the McDonald Diagnostic Criteria for Multiple Sclerosis

Clinical Presentation	Additional Data Needed for MS Diagnosis
Two or more attacks[a]; objective clinical evidence of two or more lesions	None[b]
Two or more attacks[a]; objective clinical evidence of one lesion	Dissemination in space, demonstrated by: • MRI[c] *or* • Two or more MRI-detected lesions consistent with MS plus positive CSF[d] *or* • Await further clinical attack[a] implicating a different site
One attack[a]; objective clinical evidence of two or more lesions	Dissemination in time, demonstrated by: • MRI[e] *or* • Second clinical attack[a]
One attack[a]; objective clinical evidence of one lesion (monosymptomatic presentation; clinically isolated syndrome)	Dissemination in space, demonstrated by: • MRI[c] *or* • Two or more MRI-detected lesions consistent with MS plus positive CSF[d] *and* Dissemination in time, demonstrated by: • MRI[e] *or* • Second clinical attack[a]
Insidious neurological progression suggestive of MS	One year of disease progression (retrospectively or prospectively determined) *and* Two of the following: a. Positive brain MRI (nine T2 lesions or four or more T2 lesions with positive VEP)[f] b. Positive spinal cord MRI (two focal T2 lesions) c. Positive CSF[d]

If criteria indicated are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is MS; if suspicious, but the criteria are not completely met, the diagnosis is "possible MS"; if another diagnosis arises during the evaluation that better explains the entire clinical presentation, then the diagnosis is "not MS."

[a]An attack is defined as an episode of neurological disturbance for which causative lesions are likely to be inflammatory and demyelinating in nature. There should be subjective report (backed up by objective findings) or objective observation that the event lasts for at least 24 hours.[1]

[b]No additional tests are required; however, if tests (MRI, CSF) are undertaken and are *negative*, extreme caution needs to be taken before making a diagnosis of MS. Alternative diagnoses must be considered. There must be no better explanation for the clinical picture and some objective evidence to support a diagnosis of MS.

[c]MRI demonstration of space dissemination must fulfill the criteria derived from Barkhof and colleagues[20] and Tintoré and coworkers[21] as presented in Table 2.

[d]Positive CSF determined by oligoclonal bands detected by established methods (isoelectric focusing) different from any such bands in serum, or by an increased IgG index.[56–58]

[e]MRI demonstration of time dissemination must fulfill the criteria in Table 1.

[f]Abnormal VEP of the type seen in MS.[59,60]

MS = multiple sclerosis; MRI = magnetic resonance imaging; CSF = cerebrospinal fluid; VEP = visual-evoked potential.

Reproduced from Polman and colleagues(93)

1.6.3.4 The 2010 Revision to the McDonald Criteria

In 2011, Polman and colleagues produced the 2010 revision to the McDonald criteria(92) (Figure 1.11). Polman and colleagues incorporated the simplified definition of dissemination in space proposed by Swanton and colleagues(94), particularly removing the need for gadolinium-enhancing lesions as evidence for DIS. Also brought in from the recommendations by Swanton and colleagues(94) to

remove the restriction on the follow-up MRI being done 30 days after clinical symptom onset to prove dissemination in time.

In order to prove DIS, but reduce the need for multiple scans, the panel brought in the recommendation proposed by Rovira(95) and Montalban(96) that a gadolinium-enhancing lesion alongside a T2-weighted non-enhancing lesion be sufficient evidence for DIS.

Figure 1.11. 2010 Revision to the McDonald criteria

TABLE 4: The 2010 McDonald Criteria for Diagnosis of MS

Clinical Presentation	Additional Data Needed for MS Diagnosis
≥2 attacks[a]; objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack[b]	None[c]
≥2 attacks[a]; objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a further clinical attack[a] implicating a different CNS site
1 attack[a]; objective clinical evidence of ≥2 lesions	Dissemination in time, demonstrated by: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
1 attack[a]; objective clinical evidence of 1 lesion (clinically isolated syndrome)	Dissemination in space and time, demonstrated by: For DIS: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a second clinical attack[a] implicating a different CNS site; and For DIT: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
Insidious neurological progression suggestive of MS (PPMS)	1 year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following criteria[d]: 1. Evidence for DIS in the brain based on ≥1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions 2. Evidence for DIS in the spinal cord based on ≥2 T2 lesions in the cord 3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)

If the Criteria are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is "MS"; if suspicious, but the Criteria are not completely met, the diagnosis is "possible MS"; if another diagnosis arises during the evaluation that better explains the clinical presentation, then the diagnosis is "not MS."

[a]An attack (relapse; exacerbation) is defined as patient-reported or objectively observed events typical of an acute inflammatory demyelinating event in the CNS, current or historical, with duration of at least 24 hours, in the absence of fever or infection. It should be documented by contemporaneous neurological examination, but some historical events with symptoms and evolution characteristic for MS, but for which no objective neurological findings are documented, can provide reasonable evidence of a prior demyelinating event. Reports of paroxysmal symptoms (historical or current) should, however, consist of multiple episodes occurring over not less than 24 hours. Before a definite diagnosis of MS can be made, at least 1 attack must be corroborated by findings on neurological examination, visual evoked potential response in patients reporting prior visual disturbance, or MRI consistent with demyelination in the area of the CNS implicated in the historical report of neurological symptoms.

[b]Clinical diagnosis based on objective clinical findings for 2 attacks is most secure. Reasonable historical evidence for 1 past attack, in the absence of documented objective neurological findings, can include historical events with symptoms and evolution characteristics for a prior inflammatory demyelinating event; at least 1 attack, however, must be supported by objective findings.

[c]No additional tests are required. However, it is desirable that any diagnosis of MS be made with access to imaging based on these Criteria. If imaging or other tests (for instance, CSF) are undertaken and are negative, extreme caution needs to be taken before making a diagnosis of MS, and alternative diagnoses must be considered. There must be no better explanation for the clinical presentation, and objective evidence must be present to support a diagnosis of MS.

[d]Gadolinium-enhancing lesions are not required; symptomatic lesions are excluded from consideration in subjects with brainstem or spinal cord syndromes.

MS = multiple sclerosis; CNS = central nervous system; MRI = magnetic resonance imaging; DIS = dissemination in space; DIT = dissemination in time; PPMS = primary progressive multiple sclerosis; CSF = cerebrospinal fluid; IgG = immunoglobulin G.

Reproduced from Polman and colleagues(92)

1.7 MS Treatment

As MS is a condition of aberrant immune function, all the medications aimed at treating MS are immunomodulatory in nature, either being derived from immunomodulatory cytokines (interferon-β) and other immune components (antibody therapies), other biological compounds that modulate the immune system (glatiramer acetate, corticosteroids, cladribine, vitamin D), and chemical compounds that modulate the immune system (azathioprine, mitoxantrone). Some of these act to modulate the immune system signalling pathways, most particularly interferon-β, corticosteroids and vitamin D, while others are cytotoxic chemotherapies which target immune system cells and have been found to be useful in treating MS (cyclophosphamide, cladribine)(113).

1.7.1 Interferon-β

The most-prescribed medications used in treating relapsing-remitting MS are those derived from interferon-β, a pro-inflammatory antiviral and anti-tumour cytokine produced by immune cells(59). The three interferon-β medications available in Australia, Betaferon®, Avonex® and Rebif®, are each recombinant forms of human interferon-β. Interferon-β-1b (Betaferon®) therapy was found in a Phase III randomised-controlled trial (RCT) to be significantly effective in reducing relapse rate, relapse severity, number and volume of MRI lesions, and improved change in disability(114-116). Early work demonstrated interferon-β-1a (Avonex® and Rebif®) to be effective at reducing relapse rate, lesion number and volume on MRI, and reduced progression to increased disability(117). Phase III RCTs found similar effects, with the PRISMS study finding significantly reduced relapse rate, progression and MRI-associated outcomes relative to placebo (118), while the CHAMPS study found improvements in MRI-associated outcomes and importantly, reductions in the proportion going on to clinically-definite MS from a clinically-isolated syndrome(119, 120). The INCOMIN and EVIDENCE trials found that Rebif® was more effective than Avonex®, by virtue of its being given three times weekly rather than weekly(121-126). Studies examining the effect of interferon-β on progressive courses of MS found no

efficacy(127, 128). Studies examining an oral version of interferon-β found it had no biological function or clinical effect(129).

The modes of action by which interferon-β medications realise their effects are uncertain. Its function in the body is to induce an antiviral and anti-tumour state in target cells, but such an inflammatory response is hardly desirable. Research indicates that interferon-β medications may act to increase IL-10 expression(130), inhibit T-cell migration(131), reduce antigen presentation(132) and act to restore blood brain barrier integrity(133). Also, some research has suggested it may act against herpesvirus replication and the modes by which herpesviruses manifest in MS pathology(134, 135) (see section 1.8.5).

1.7.2 Glatiramer acetate

Another major treatment for MS is glatiramer acetate, also known as copolymer 1, and marketed under the name Copaxone®, and administered in 20mg subcutaneous injections daily(113). RCTs have found glatiramer acetate to significantly reduce relapse rate and progression to increased disability(136, 137). The mode by which glatiramer acetate exerts its therapeutic effects is unclear but it is thought it may act as a competitive agonist for MHC class II receptors which would otherwise bind MBP(113, 138-140). A related medication, dirucotide or MBP-8298 is a synthetic peptide of 17 amino acids of MBP which is thought to act as a target for immune cells to lure them away from MBP, and is presently undergoing Phase III RCTs(141).

1.7.3 Monoclonal antibody therapies in MS

A number of therapies have been developed which target specific proteins which are solely or principally found on immune cells of relevance to MS. By targeting these proteins, they can induce targeted lysis of immune cells, induce their sequestration away from the CNS, prevent their entering the CNS at all(142).

Among the most recent medications for MS, natalizumab is a monoclonal antibody against the α4-integrin receptor used by lymphocytes to extravasate from circulation into the CNS(143). Two Phase III RCTs have demonstrated natalizumab to be effective in treating MS, with treatment yielding significant reductions in relapse rate and progression to increased disability, as well as reducing lesion number on MRI(144) relative to placebo, and relative to interferon-β-1a treatment(145). There has been some evidence of increased risk of herpesvirus reactivation, possibly due to reduced immune surveillance due to the effects of natalizumab(146).

Rituximab is a monoclonal antibody targeting the CD20 receptor found on B-lymphocytes. Originally approved to treat B-cell non-Hodgkin's lymphoma, due to its cytolytic effects on B-cells, in light of the well-known oligoclonal bands in the CSF of MS, research into use of rituximab in treating MS was undertaken(143). Administered by intravenous infusion, rituximab has been shown in Phase I(147) and Phase II(148) RCTs to reduce relapse frequency and MRI-associated outcomes relative to placebo, though clinical effects were less impressive among PPMS patients(149).

Alemtuzumab is a monoclonal antibody targeting the surface receptor CD52 found on mature lymphocytes, monocytes and dendritic cells, but importantly not the haematopoietic progenitors. Originally developed to treat lymphocytic leukaemia and later also approved for use as a transplant immunosuppressant, alemtuzumab has been developed as a therapy for MS. An annual intravenous infusion, alemtuzumab targets the CD52 protein and, by recruiting complement, induces cytolysis and elimination of the targeted cells. This results in a potent and sustained lymphopoenia, with CD4 T-cells depletes for a median of 61 months, and CD8 T-cells depleted for a median of 30 months(150). This immune cell depletion is therapeutic against the autoimmune MS, but does not manifest in any significantly immunocompromised state(150). Moreover, recent analysis suggests that the reconstituted immune cell population following treatment is more conducive to remyelination and

immune repair which may contribute to its effects in reducing disability(151). Phase II RCTs have found alemtuzumab to be effective in reducing relapse frequency and progression(152, 153). At present, two Phase III RCTs are in progress.

Daclizumab is a monoclonal antibody which targets the α-subunit of the IL-2 receptor found on activated T-lymphocytes(143), and in so doing prevents IL-2-induced T-cell proliferation. An intravenous infusion, early trials indicated daclizumab was effective at reducing MRI-associated outcomes, relapse rate and progression to increased disability(154). Phase II trials found similar effects, with patients unresponsive to interferon-β therapy having significantly reduced enhancing lesions on MRI and improvements in clinical disability scores(155, 156). Presently Phase III RCTs are underway(157, 158).

1.7.4 Fingolimod

Derived from a metabolite of the fungus *Isaria sinclarrri*, fingolimod is an analogue of sphingosine, binding to the sphingosine-1-phosphate receptor on lymphocytes, inducing the sequestration of immune cells in lymph nodes and thus, out of the CNS(159, 160). An oral medication taken daily, fingolimod has been demonstrated in phase III RCTs to reduce relapse rate, improve MRI measures and reduce progression to increased disability, relative to placebo(161).

1.7.6 Other immunomodulatory agents

Corticosteroids have long been used for their anti-inflammatory effects on a range of conditions, most particularly arthritis. It is thus no surprise to find them being utilised for these anti-inflammatory effects in treating MS(162, 163), most particularly methylprednisolone. High-dose corticosteroids are used in treating severe relapses, these administered by intravenous infusion; however some physicians prescribe lower dose corticosteroids to realise a reduction in inflammation which might lead to relapse.

Mitoxantrone was initially developed as an anti-cancer chemotherapy. A type II topoisomerase inhibitor which, in interfering with DNA replication and thence cell replication, mitoxantrone is useful in blocking proliferation of inflammatory immune cells(113). Consequently, this drug is a potent, but toxic immunomodulator. Moreover, its mode of administration by intravenous infusion, requiring in-patient admission, alongside the side-effects inherent in its mode of action, restricts its use to only the most severe relapses. RCTs have found that mitoxantrone treatment yielded significantly reduced relapse rates and improved MRI-associated outcomes, and progression to increased disability(164, 165). In contrast to many other therapies, mitoxantrone has shown some efficacy in progressive courses of MS(166).

Azathioprine is a pro-drug of the standard cytotoxic medication 6-mercaptopurine, a purine synthesis inhibitor(113). Once inside target cells, the drug is metabolized to the active form, which blocks further DNA synthesis. RCTs have demonstrated improvements in relapse rate and reduced progression to increased disability(167), and studies suggest azathioprine may be useful as an adjuvant along with interferon-beta medications(168-170).

Cyclophosphamide (Cytoxan®) is a pro-drug, converted in the liver to the active form, which forms permanent crosslinks between DNA strands, preventing DNA replication and resulting in apoptosis(113). Given its non-specific targeting of proliferating cells, resulting in typical chemotherapy side effects, as well as its intravenous mode of administration, this medication is restricted for use in only the most severe relapses. Early studies have shown cyclophosphamide to be efficacious in reducing relapse rate(171).

Cladribine® (2-chlorodeoxyadenosine) is a purine analogue which, when incorporated into DNA acts to terminate DNA replication(113). Early studies demonstrated cladribine therapy markedly reduced

lymphocyte numbers in serum(172, 173) and RCTs have shown cladribine to markedly reduce or terminate progression to increased disability relative to placebo(174, 175) and to improve MRI-determined outcomes(175-177), though other studies found no significant difference in change in disability between treatment and placebo groups(178) and no effect on changes in brain volume(179). Among the first oral medications to be approved for use in treating MS(175), its relatively limited side effects make this a promising addition to the MS treatment arsenal.

Laquinimod is a novel immunomodulatory agent, the precise mode of action of which is unclear, but is thought to include interfering with immune cell extravasation into the CNS, inducing a Th2 immune profile in target immune cells, and contributing to remyelination(180, 181). An oral medication taken daily, Phase II RCTs have shown it to be effective in reducing MRI-associated outcomes relative to placebo, though no improvement in relapse rate or disability have been found(182, 183).

Teriflunomide is an active metabolite of leflunomide, an immunosuppressant medication which blocks pyrimidine synthesis in proliferative cells and thus, blocking replication(180, 184) and antibody production(180). A daily oral medication, Phase II RCTs have found significantly reduced relapse rate and improvements in MRI-associated outcomes relative to placebo(185). Phase III RCTs are in progress(180).

Dimyethyl fumarate medications are a novel class of oral medication, previously investigated for treatment in psoriasis(186), which have been found to induce production of anti-inflammatory cytokine production in immune cells, induce apoptosis in activated T-cells, and downregulate binding receptors which reduce extravasation of immune cells into the CNS(187). Early studies demonstrated significant reduction in MRI-determined lesion number and volume(187). A Phase II RCT showed significant

reductions in the number of enhancing lesions on MRI and reduced relapse rate, relative to placebo(188). Presently Phase III RCTs are underway(189, 190).

1.8. MS aetiology & modulators of clinical course

1.8.1 Genetics

1.8.1.1 HLA-DR

The HLA genomic region encodes the major histocompatibility complex (MHC), a key protein complex involved in identification of "self" on cells (MHC Class I), as well as in antigen presentation (MHC Class II) between antigen presentation cells (APC) and lymphocytes(59) (see section 1.4.2). Some of the strongest associations among genetic factors in MS have been for genes of the HLA-DRB region(191-194), encoding the β-subunit of the MHC heterodimer. This is not unexpected, given certain alleles may yield MHC complexes predisposed to binding and presenting 'self' proteins, inducing an autoimmune response on those proteins(59, 192, 193). Potent associations have now been found for alleles of HLA-DRB1, HLA-DQA1, and HLA-DQB1, though the magnitude and even direction of these effects can vary between northern and southern European-descent populations(195, 196).

1.8.1.2 Vitamin-D –related genes

A number of genes relevant to vitamin D have been found to be associated, not merely with MS, but with other autoimmune disorders(197). Variations in the key proteins involved in vitamin D's activity, including vitamin D binding protein (VDBP), 1α-hydroxylase and vitamin D receptor (VDR), could markedly affect the ability of vitamin D to exert its effects (see section 1.8.2). While no significant associations have been found for polymorphisms of VDBP(198, 199), there is some evidence to suggest that polymorphisms in 1α-hydroxylase(200) or VDR(201-203) may be significantly associated with increased risk of MS, or show interactive effects with other risk factors(198, 204, 205).

1.8.1.3 Other genes

Another gene thought to affect MS risk is the T-cell receptor (TCR) gene(195). This is not surprising, given TCR is the other side of the MHC Class II couple via which an autoimmune response would be

initiated(59). Another protein on the surface of T-cells thought to be associated with MS is the Cytotoxic T-lymphocyte antigen 4 (CTLA-4). Binding of this T-cell co-receptor to the corresponding receptor on APCs induces anergy in T-cells, making it a critical part of the immune system's 'check' against self-targeting immune cells. It may be that certain alleles of this protein may make it less capable of properly inducing anergy in autoreactive immune cells, and accordingly, some studies have demonstrated an association with MS risk(195). Recent studies have suggested some interaction between HLA-DRB1 alleles and CTLA-4 in MS risk, further substantiating a role for CTLA-4(206).

1.8.2 UVR & vitamin D

Among the various environmental factors evaluated for their association with MS, some of the strongest and most consistent have been with personal ultraviolet radiation (UVR) exposure and vitamin D. Research into vitamin D and MS was to some extent sourced with the aforementioned latitudinal gradient hypothesis (see section 1.3.2), given the strong correlation between higher latitude and reduced winter sun exposure and thence, lower winter vitamin D. Indeed, the association between MS frequency and latitude have engendered various interpretations of the mode of action, including genetics(37, 38, 207-210), UV and vitamin D(211-215), the distribution of Epstein-Barr virus infection(216-218), or combinations thereof(46, 47, 219-221).

Of these, however, vitamin D is the strongest contender. The major circulating metabolite used for assessment of vitamin D status is 25-hydroxyvitmain D (25(OH)D), while the active form is 1,25-dihydroxyvitamin D ($1,25(OH)_2D$)(222). As described in Chapter 4, findings are strongly complimentary between the latitudinal gradient hypothesis and the relationship between past personal UVR exposure, during childhood(213, 223) and even while in-utero(224), and subsequent risk of MS, and childhood vitamin D intake(225) and risk of MS. Similarly, the significant differences in current vitamin D intake(226, 227) and serum levels of vitamin D metabolites(228-230) between relapse and remission samples and by level of disability(231), and more recently, from prospective cohort studies

evaluating vitamin D levels and subsequent hazard of relapse(232, 233), provide strong evidence in favour for vitamin D in MS, and as being the principal environmental mediator of the latitudinal gradient. Among these latter cohort studies is a work evaluating the relationship between levels of serum 25-hydroxyvitamin D (25(OH)D) and subsequent risk of relapse, the results of which were published in 2010(232) and are presented in Chapter 5.

A detailed discussion of vitamin D physiology and particularly its immunomodulatory effects are presented in Chapter 4. Here follows a summary of the epidemiological evidence linking personal UVR exposure and vitamin D with MS risk and clinical course.

1.8.2.1 Season of birth and MS

Season of birth studies have found significantly higher rates of births in spring months and thus, later gestation during winter months, among MS patients than would be expected by chance alone (Appendix 1A Table 1). This effect of season of birth was recently demonstrated to be entirely a manifestation of UV exposure of the mother while the child was in-utero(224). While season of birth was significantly associated with subsequent MS risk, adjustment for region of birth and first-trimester UV exposure of the mother abrogated the season of birth association(224).

Season of birth has also been associated with a delayed onset of MS, with those born in spring having an earlier onset of MS relative to those whose gestation occurred in spring/summer(234). Season of birth has been found to correlate with greater disease progression to increased disability, with those born in winter, and thus gestating during spring and summer, having significantly slower progression(235), though this was not replicated elsewhere(236).

1.8.2.2 Season and MS

Several studies have examined the seasonal changes in clinical and MRI-detected exacerbations: using a cross-sectional analysis of a prospective cohort, Tremlett and colleagues(237) found a significant seasonal variation in monthly relapse rate, with significantly higher rates in winter months, relative to

summer. Auer and colleagues(238) found significantly more MRI disease activity occurred during spring and early summer, relative to autumn. Embry and colleagues(239) re-evaluated this association, suggesting that the seasonal changes in MRI activity were a manifestation of changes in serum 25(OH)D (Table 1.1).

Table 1.1. Season and clinical course

Study	Study type	Sample	Relapse/remission	MRI
Auer 2000(238)	Prospective cohort	53 RRMS or SPMS		**Significantly more MRI-detected disease activity in Winter/spring vs. Summer/autumn ($p<0.006$)**
Killstein 2002(240)	Prospective cohort	28 MS	No significant seasonality in relapse ($p>0.05$)	No significant seasonality of active MRI lesions ($p=0.180$) or active MRI scan ($p=0.283$)
Tremlett 2008(237)	Prospective cohort	199 MS (142 RRMS)	**Significantly greater relapse rate in winter vs. summer months ($p=0.018$)**	

1.8.2.3 UV exposure and MS

Evaluating the relationship between UV exposure and subsequent risk of MS is necessarily imprecise, relying on subjects to approximate past sun exposure behaviours, often many years prior. This is itself subject to some amount of measurement error and recall bias is a particular concern. However, the several studies done which evaluated childhood/adolescent exposure to UV and subsequent risk of MS are fairly consistent in their findings (Appendix 1A Table 2), demonstrating significant inverse relationships between childhood UV exposure, measured by time and level of sun exposure.

UV exposure also has an inverse relationship with MS clinical course (Table 1.2), with progression and exacerbation rate showing a significant inverse correlation with UV exposure.

Table 1.2. UV exposure and clinical course

Study	Study type	Sample	Relapse/remission	Progression
van der Mei 2007(241)	Case-control	136 MS, 272 age/sex-matched controls		**Significant inverse correlation btw UV exposure and EDSS (p<0.0001)**
Tremlett 2008(237)	Ecological	199 MS (142 RRMS)	**Monthly relapse rate inversely correlated w/ 1.5/12 lagged UV (p=0.046)**	

1.8.2.4 Vitamin D intake

Intake of vitamin D has been inconsistent in its association with MS. In the two pooled analyses (Table 1.3) of past dietary intake of vitamin D-rich foods and supplements, only vitamin D supplements had a significant inverse association with MS risk(225), though this was not replicated in the subsequent study(242). What was consistent between the two studies is that vitamin D-rich foods had no effect on risk of MS.

Table 1.3. Vitamin D intake: cases vs. controls

Study	Study type	Sample	Vitamin D	RR/OR	Comment
Munger 2004(225)	Prospective pooled study NHS1 and NHS2	173 MS	Diet and supplement, baseline and prosp. Cumulative	**Pooled age-adj. RR baseline high vs. low: 0.67** **p=0.03**	Supplement only – no effect from diet
Munger 2010(242)	Prospective pooled study NHS1 and NHS2	379 incident MS (NHS1); 67 prevalent MS (NHS2)	Diet and supplement during adolescence	No effect from diet. >=400IU/day supplement RR: 0.73 (p=0.11)	Non-sig effect from supplement, no effect from diet

1.8.2.5 Vitamin D treatment

There have been only 2 studies(226, 227) (Table 1.4) which evaluated the effect of vitamin D supplementation on MS clinical course, and these had very small samples and relatively short follow-up,

as well as being unblinded. Both studies showed a positive effect of vitamin D supplementation, both in reduced relapses(226, 227) and less progression to increased disability(227); however the study design does not allow for a demonstration of causality.

Table 1.4. Vitamin D treatment and clinical course

Study	Study type	Sample	Tx	Relapse/remission	Progression
Goldberg 1986(226)	Case-crossover trail	16 MS	Calcium, magnesium supplements and 5000IU/day vitamin D x 1-2yrs	**Significantly reduced # relapses w/ Tx ($p<0.01$)**	
Burton 2010(227)	Prospective unblinded 52-wk RCT	25 Tx, 24 control	40,000 IU/d x 28/52, 10,000IU/day x 12/52, 0IU/day x 10/52 vs. placebo	ARR during trial ($p=0.09$); Proportion with relapses during study ($p=0.09$)	**Proportion w/ increased EDSS ($p=0.019$)**

1.8.2.6 Serum vitamin D metabolites: Cases vs. controls

There have been a number of studies which compared the levels of serum levels of vitamin D metabolites, either 25(OH)D (Appendix 1A Table 3) or $1,25(OH)_2D$ (Appendix 1A Table 4), between cases and controls. There is some evidence of a difference in the associations for both metabolites by MS course(229), race(228) or sex(228). Also, a study by Hanwell and colleagues(243) prospectively followed a cohort of 125 children after a clinically-isolated syndrome for one year, finding that the 20 children that developed MS had significantly lower serum 25(OH)D than those who did not ($p=0.029$).

1.8.2.8 Serum vitamin D metabolites and MS clinical course

Studies which examine the relationship between serum levels of 25(OH)D and $1,25(OH)_2D$ with clinical course fall into two groups: cross-sectional designs(229-231, 241, 244, 245) which compare levels during periods of relapse and remission, or by measures of disability, and cohort studies which evaluate cases' clinical courses prospectively(232, 237, 246, 247) or retrospectively(248) as relates to serum levels.

Serum 25(OH)D (Appendix 1A Table 5) samples collected during relapse are almost invariably significantly lower than those collected during remission(229, 230, 232, 244, 246, 248). Serum $1,25(OH)_2D$ showed a similar association (Appendix 1A Table 6), with significantly lower levels found in relapse samples compared to remission samples(229, 230, 248).

Several studies evaluated serum vitamin D metabolites with the frequency or hazard of relapse(232, 237, 248). These studies are consistent in their findings of strong, inverse associations between serum 25(OH)D and the risk of relapse. Also, a prospective cohort study(233) of 110 paediatric MS patients found a significant inverse relationship between serum 25(OH)D and the risk of relapse, each 10ng/mL increase associated with a 34% reduction in the rate of subsequent relapse ($p=0.024$), these findings are quite similar to those observed among adult-onset MS(232).

1.8.2.9 CSF 25(OH)D and MS

Unfortunately, there are much fewer studies examining the relationship between levels of vitamin D in the CSF/CNS and MS. Indeed, only one study(245) was found which evaluated 25(OH)D levels in the CSF, finding no significant difference between cases and controls nor any correlation with MS exacerbations, either clinical or on MRI.

1.8.3 Acute Infection

Research investigating the link between acute infection and relapse in MS has consistently shown a strong relationship, with rate ratios and odds ratios averaging about 2.8(249-254). Much of this work has sought to identify the agent at fault, principally focused upon upper respiratory tract infection (URTI)-associated pathogens. Going back to the initial work by Sibley and colleagues(249), continuing to the present, both epidemiologic and serological studies have shown that the period 1-2 weeks before infection symptom onset to 2-5 weeks after is a period of high risk for having a relapse, relative to outside this period. Why this time period around acute infection is so important has not yet been deduced, though mechanisms of immune triggering(249, 250, 255), immune modulation(252, 253), molecular mimicry(251), and direct infection of the CNS(254) have been suggested. It is hypothesized

that the pathogens causing acute infection induce relapse, either by direct or indirect effects upon the CNS, but no one has yet proposed a pathway by which a transient exposure to a pathogen in the respiratory tree might manifest in altered neuropathology in the CNS. The finding that susceptibility to acute infection is affected by season and vitamin D status(256), and that vitamin D affects relapse risk(232, 237), is suggestive that the two might be a common outcome of one another (see Chapter 4).

1.8.4 Childhood infection

Some intriguing findings have suggested that acute infections during childhood might modulate subsequent risk of relapse(257, 258), such that increased exposure to pathogens during childhood may yield a reduced risk of aberrant immune behaviour later in life. This phenomenon, reflective of the hygiene hypothesis(259), may indicate that there exists a period in life wherein the immune system is more tolerant and efficient at responding to exposure to otherwise benign pathogens, whereas exposure later in life may result in a more aggressive immune response, with autoimmune collateral damage resultant. There is some evidence that this tolerance may relate not merely to age but also to season and vitamin D status at exposure (see Chapter 4).

1.8.5 Human herpesviruses

Some of the strongest associations among infectious factors with MS risk have been exposure to and response against human herpesviruses, particularly Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV-6). These herpesviruses are ubiquitous in the human population(260, 261), but are especially frequent in persons with MS, with virtually 100% of cases having been exposed at some point in their lives(261). However, given the ability of most human herpesviruses to infect and establish latency in neuronal and glial cells of the central nervous system(262-268), including oligodendrocytes, astrocytes and microglia, as well as infiltrating immune cells(269), work has been done evaluating EBV(221, 270) and HHV-6(271), as well as herpes simplex virus (HSV)(272-286), varicella zoster virus (VZV)(272, 273, 275, 276, 279, 281-284, 287-295), and cytomegalovirus (CMV)(272, 274-276, 281, 282, 284, 295-297) for their roles in MS; work evaluating human herpesviruses 7 and 8 have not found any

significant associations(261). Of the herpesviruses, however, the strongest and most consistent of these have been for EBV and HHV-6.

As MS is a disease of the central nervous system, identification of virus in a greater proportion of case vs. control brains and/or cerebrospinal fluid, and particularly in and around MS-associated lesions would be potent evidence in favour of an aetiological role. Unfortunately, given the difficulty in obtaining brain and CSF samples, there are few studies evaluating such samples. Pathology studies have found HHV-6(273, 298-302) more frequently in MS brains, particularly in and around MS lesions(273, 298, 300-303); others(304) found no excess of HHV-6 in MS brains, however. For EBV, fewer pathology studies have been done, and none have found any greater frequency of EBV in MS vs. control brains(305-308). Studies of the CSF are relatively more frequent, given patients needn't be deceased, though the necessity of a lumbar puncture does make these samples still difficult to obtain. For HHV-6, some studies demonstrated serological(309-313) and viral load(295, 314) markers of HHV-6 infection in the CSF of MS patients, while others found no greater evidence of virus, either serological or viral load, in the CSF of cases vs. controls(275, 276, 290, 309, 315-321). For EBV, some studies have demonstrated serological evidence of EBV in the CSF(322-324), while for others, no excess was found in MS CSF vs. controls(275, 276, 290, 295, 306, 309, 318, 319, 325, 326)

A much greater number of studies have been done evaluating levels of serological and viral load markers of infection in serum, given the ease with which these samples can be collected. Moreover, these studies can evaluate the association between levels and clinical course, comparing samples collected from multiple time points within the same individual, during relapse and remission, and assess any associations.

The study by Levin and colleagues(327) is among the strongest points of evidence linking EBV and MS, given its prospective nature. Herein, samples collected years before the onset of MS were evaluated for their association with subsequent conversion to MS, with higher titres correlating significantly with subsequent MS. A more recent study by Lunemann and colleagues(328) supports those by Levin, with anti-EBV-EBNA titres significantly predicting subsequent conversion to MS. Similarly, this year's study by Munger and colleagues(329) found anti-EBV-EBNA IgG to be significantly associated with subsequent conversion to MS.

1.8.5.1 Serological exposure to HHV-6 and EBV

As shown in Appendix 1A Table 7 and 8, there are a number of studies comparing titres of IgG against HHV-6 and EBV between MS cases and controls. For both viruses, exposure is very common, with many studies finding near 100% exposure for HHV-6(278, 311, 312, 321, 330, 331) and EBV(257, 332-338). For HHV-6, a number of studies have found either higher frequencies of detection(309, 312, 339, 340) or higher titres(278, 341-343) in cases vs. controls; however a number of studies find no difference in either(278, 310, 311, 316, 321, 330, 331, 344, 345). For EBV, some studies find higher frequencies of detection(274, 277, 286, 337, 346-350) and/or higher titres(257, 278, 332, 334, 335, 345, 347, 348, 351) in cases relative to controls, though here again other studies found no difference(257, 278, 321, 336, 338, 340, 345).

For both viruses, a comparative paucity of studies has evaluated the relationship between serological exposure to HHV-6 or EBV and clinical course, instead focusing on markers of reactivation, primarily viral load. Villoslada and colleagues(345) evaluated disease course covariates against anti-EBV-EBNA IgG, finding titre correlated inversely with EDSS at the time of measure. Farrell and colleagues(334) found anti-EBV-EBNA IgG titre correlated positively with MRI activity, though no association was found for anti-EBV-VCA IgG. In light of this paucity of such research, presented in Chapter 7 is work

evaluating the role of serological markers of exposure to EBV and HHV-6 and their relationship with clinical course.

Studies looking at the marker of recent exposure to EBV (anti-EBV IgM) found no greater frequency among cases vs. controls (Table 1.5). This is not surprising, since primary exposure to EBV occurs at or before adolescence in most populations.

Table 1.5. Serum anti-EBV IgM and MS onset and clinical course

Author	Cases	Assay & antigen	Type, period	MS Onset	Clinical course
Soldan 1997(331)	36 MS, 31 OND, 21 OID, 14 controls	IFA, EBV-EA	Case-control	No difference between cases and controls	
Villoslada 2003(345)	49 RRMS, 50 SPMS, 53 CIS, 50 controls	ELISA, EBV-VCA	Case-control	No difference in EBV-VCA	
Buljevac 2005(333)	54 RRMS	ELISA –EBV-VCA	Prospective cohort (mean 1.7yr)	3/188 samples from 3 subjects positive	
Farrell 2009(334)	50 MS, 50 CIS	Quantitative chemoluminscent assay: EBV-VCA	5-yr prospective	No subjects with evidence of EBV reactivation	
Khaki 2011(340)	61 MS, 60 controls	ELISA, IFA	Case-control	No difference between cases and controls	

1.8.5.2 Serological reactivation of HHV-6 and EBV and MS aetiology and clinical course

As in Appendix 1A Tables 9 and 10, a number of studies have evaluated the frequency and titre of serological markers of reactivation for both HHV-6 and EBV. For HHV-6, several studies found little evidence of serological reactivation in any subject(309, 316, 321, 344, 352). Of those finding evidence of serological reactivation, some found either greater occurrence(310, 331, 340, 342, 353) or greater titres(341, 345), but others found no difference between cases and controls(315, 348). For EBV, findings have been more mixed, with some studies demonstrating higher detection(277, 333) or titre(333, 351) in cases than controls, while others found no differences(274, 335, 345, 348).

Here again, relatively few studies evaluated the relationship between serological markers of reactivation and clinical course. What little that has been done is not very revealing. Villoslada and colleagues(345) found that anti-HHV-6 IgM correlated inversely with EDSS and disease duration, suggesting that HHV-6 reactivation played a greater role earlier in the course of MS. For EBV, Wandinger and colleagues(274) found significantly greater frequencies of reactivation in relapse vs. remission samples, while Buljevac and colleagues(333) found no difference.

In Chapter 8 is presented our work evaluating the frequency of HHV-6 serological reactivation and its relationship with MS clinical course.

1.8.5.3 Serum HHV-6 and EBV viral load and MS aetiology and clinical course

Viral load is a more sensitive marker of viral reactivation, by virtue of its use of signal amplification by polymerase chain reaction (PCR), and thus it is more preferred for evaluating viral reactivation than is serology. While this is useful when done in temporal proximity to the outcome of interest, particularly in relation to relapse, it is limited by its being detectable only at the time of reactivation. Thus, where a sample is taken shortly after the resolution of a reactivation, a negative result for viral load would result, whereas serology would afford a broader, albeit less sensitive window of detection.

As in Appendix 1A Table 11, for HHV-6, as with serology, a number of studies have found little or no evidence of viral reactivation by viral load(275, 276, 309, 312, 316, 320, 348, 352, 354-358). This may be due to the aforementioned limitations of viral load measure of reactivation. Of those finding evidence of viral reactivation, however, a number find greater frequency(331, 359-368) in cases than controls; others find no difference, however(135, 317, 320, 356-358, 369, 370). For EBV (Appendix 1A Table 12), again a number of studies found little or no viral load in any samples(275, 276, 312, 334, 348). Of those that did, none found any significant difference between cases and controls(365, 370, 371).

For studies evaluating the relationship between viral load and clinical course, those few studies did so in a cross-sectional fashion, comparing frequencies and magnitudes between relapse and remission samples. For HHV-6, Berti and colleagues(363) found significantly higher frequencies of detection in relapse vs. remission samples; similar findings were realised by Alvarez-Lafuente and colleagues(365, 366) and others(370, 372), though others found no differences(135, 320, 360, 368). Others found that magnitude of viral load correlated positively with relapse(135, 362). For EBV, Wandinger and colleagues(274) first demonstrated that EBV DNA was more frequently detected in relapse vs. remission samples, this also found by Hollsberg(370); others found no significant differences, however(333, 365, 372).

1.8.5.2 Modes of action for HHVs and MS

Potential modes by which human herpesviruses could manifest in neurotoxic inflammation are varied, and include direct and indirect effects.

The simplest and more direct is lytic replication of virus inside cells of the immune and nervous systems, including glial cells within the CNS, as well as peripheral immune cells which have entered the CNS. As noted previously, herpesviruses establish latency inside both CNS cells and peripheral immune cells, with reactivation occurring over time. Such reactivation in the CNS would induce local response by microglia and astrocytes, potentially recruiting peripheral immune cells into the CNS and thus exacerbating inflammation. Infection of astrocytes by HHV-6 induced deregulated glutamate uptake(373), a key function of the astrocytes, this potentially yielding neurotoxic conditions. HHV-6 has been found to indirectly induce apoptosis of oligodendrocytes, even after removal of HHV-6 altogether, with some component in the supernatant from infected cells inducing oligodendrocyte death(374).

Molecular mimicry is also a potential mode of action, whereby the immune response to a viral protein targets an immunologically-similar host protein. A number of studies have demonstrated plausible modes by which similarities between HHV-6 and EBV antigens with host proteins, such as HHV-6 and myelin basic protein (MBP)(375), and EBV-EBNA and MBP(323, 376-379), and EBV and alpha B-crystallin(380), could result in autoimmune attack of these host proteins.

The membrane receptor by which HHV-6 gains entry to target cells, CD46(381), is an immunomodulator involved in complement activation and regulation. In binding to CD46, HHV-6 induces signaling pathways which induce production of cytokines, including IL-1β and IL-17(382). CD46 has been found to be over-expressed as a soluble protein in persons with MS(383), possibly reflecting a mode of immune dysregulation that could result in some pathology.

EBV and HHV-6, like many herpesviruses, have virally-encoded immune protein analogues that act to disrupt host immune response and could act to manifest in local inflammation. HHV-6 encodes a viral analogue of the host CCL2 chemokine, which acts to recruit monocytes and basophils, acting as an agonist for the CCR2 receptor and thus reducing the ability of CCL2 to bind its receptor and exert its effects(384). EBV encodes a viral analogue of the host IL-10(385, 386). This vIL-10 binds to the IL-10 receptor, inducing some of its effects but not others(385, 386), manifesting in aberrant immune function which, while beneficial to viral reproduction, could yield some of the immunopathology found in MS. Hayes suggested a novel hypothesis whereby EBV-encoded vIL-10 might interact with low serum 25(OH)D in winter, with the two immunomodulatory effects combining to manifest in increased inflammation and leading to relapse (see Chapter 4). Another point of possible interaction is indicated by recent findings that EBV-EBNA3 interferes with vitamin D genomic effects(387), which might further contribute to immune dysregulation, regardless of vitamin D status. Also, work has suggested

some interaction between anti-EBV-EBNA titre and HLA-DRB1*15 and MS risk(388), or EBV and HLA-DRB generally and MS risk(389).

Additionally, herpesviruses have the ability to transactivate one another(261, 390-392), either in part or in total, allowing the possibility that observed associations with some viruses, such as HSV, VZV and CMV, may reflect transactivation of EBV and/or HHV-6. A further point of interest is herpesvirus transactivation of human endogenous retrovirus (HERV)(393-398). HERVs are residuals of retrovirus genomes incorporated into the germ line of long-distant ancestors, and thus are present in all the cells of the body. While not replication-competent, partial transcription and translation of viral proteins can occur(391, 399), some of which have been found to be highly neurotoxic and inflammatory(399-401) and some studies have found these HERV proteins to be present in the CNS of persons with MS(295, 304, 402). Moreover, studies have found that in addition to direct transactivation by herpesviruses, pro-inflammatory cytokines can induce expression of HERV proteins in glial cells of the CNS(403, 404), allowing the possibility that inflammation due to herpesvirus reactivation in the CNS might indirectly induce the expression of HERV proteins, resulting in further inflammation.

1.8.6 Tobacco smoking

Within MS research, in addition to a risk for disease(405-418) or risk for conversion to definite MS(419), exposure to cigarette smoke has been linked to a worse clinical course, including increased lesion volume(420, 421), greater brain atrophy(420, 421), greater rates of progression to increased disability(420-424), as well as increased risk of progression to(413, 420, 423, 425), or presentation with a more progressive course(420, 425). Additionally smoking has been associated with a transient deterioration in patients' cognitive and/or motor function, either during existing exacerbations(17), or generally in comparison to non-smoking controls(426), these effects suspected to be due to the effects of nicotine on the CNS and vascular system(426). Besides MS, the role of smoking as a casual factor has been discussed for a number of autoimmune conditions, including rheumatoid arthritis(412, 427-429),

systemic lupus erythematosus(427, 428), Graves' disease(427, 429), Crohn's disease(412, 429), and ulcerative colitis(412, 429), as well as others(427).

While there is significant literature to suggest that smoking plays a role in MS aetiology and clinical course, the biological mechanisms by which this might occur are less clear. Tobacco and the resultant smoke from cigarettes contains over 4,500 compounds and chemicals, including a number of known carcinogens and other toxic substances(430). Among these substances are some with known immunomodulatory effects, most particularly nicotine, though the immunomodulatory effects of all the compounds are not known. Nicotine has been found to modulate immune cell activation and differentiation and responsiveness to stimuli, as well as to interfere with the function of activated immune cells, including cell-mediated lysis of target cells and the production of pro-inflammatory cytokines, including IL-2, IL-12, IFN-γ, TNFα, and MIP-1α(431, 432). Interestingly, nicotine has been found to bind to and stimulate the T-cell receptor (TCR) on T-cells which, in the absence of costimulatory molecules, induces a state of anergy in these cells(431).

1.8.7 Other: pregnancy and stress

It has long been recognised that the risk of acute exacerbation is significantly reduced during pregnancy(433, 434), while in the post-partum period, the risk is significantly greater(434, 435). A study evaluated the cytokines produced by stimulated peripheral blood monocytes (PBMC) taken from women during and after their pregnancy, found that PBMCs taken during pregnancy showed a T_h2 response, whereas those taken after, showed a markedly T_h1 response(436). The mechanisms by which pregnancy modulates risk of relapse in MS is unclear but may involve changes in hormone levels(437-441), or changes in circulating vitamin D levels and in vitamin D metabolism(442-444) (see Chapter 4).

Mental and emotional stress has been associated with a worse clinical course in MS, with a significantly greater risk of relapse after stressful life events(445). The mechanism by which stress may manifest in exacerbation of MS is necessarily complex, given the complexity of emotion. However, it is being

increasingly recognised that there is a powerful interaction between emotional state and the innate and adaptive immune system(446). Also, human(447) and animal(448, 449) models have found significantly altered immunological parameters in depressed patients. There is also evidence to suggest some interaction between stress and vitamin D-associated pathways, which may account for the some of the associations of stress with MS (see Chapter 4).

1.9 Structure of this thesis

This thesis is comprised of several analyses of somewhat varied topics, but each fall within two major areas of MS research: geoepidemiology (Chapters 2 and 3) and determinants of clinical course (Chapters 4-8).

In Chapter 2 is presented an analysis of the trends in the epidemiology of MS in the Greater Hobart area of Tasmania, specifically the prevalence between 1961 and 2009, and incidence and mortality rates between 1951-61 and 2001-9. The distribution of these measures is evaluated and suppositions as to the determinants of the trends and what they may portend for the future discussed.

In Chapter 3 is presented the results of a meta-analysis of MS prevalence as relates to latitude at the global, supra-regional and regional levels. The nature of the associations at these levels is evaluated and the drivers of the global and regional gradients discussed.

In Chapter 4 is presented a review on the physiology, immunology and epidemiology of vitamin D and related exposures on MS onset and clinical course. Additionally, possible ways in which vitamin D may be mediating or modulating other aetiologic pathways are discussed.

In Chapter 5 is presented the results of an analysis of the role of the diagnostic vitamin D metabolite 25-hydroxyvitamin D in predicting subsequent hazard of relapse. For this, the biannually-measured 25(OH)D is used, as well as a season-adjusted estimate, and an estimate of the 25(OH)D within individuals at monthly levels.

In Chapter 6 is presented the results of an analysis of the relationship between reported interferon-β use in the 6-month interval preceding each review and the measured 25(OH)D at that review. Additionally, the interaction between reported interferon-β use and 25(OH)D in predicting subsequent hazard of relapse is presented.

In Chapter 7 is presented the results of an analysis of the relationship between measures of serological exposure to human herpesvirus 6 and Epstein-Barr virus and subsequent clinical course, specifically hazard of relapse and change in disability as measured by EDSS and MSSS.

In Chapter 8 is presented the results of an analysis of the frequency of serological reactivation of HHV-6 and its relationship with clinical course, specifically relapse occurrence and disability.

Finally, in Chapter 9 is presented an overarching discussion of the material presented in this thesis.

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Appendix 1A. Tables for seasonal, UV, vitamin D and measures of herpesvirus association with MS and its clinical course

Appendix 1A Table 1. Season/month of birth and MS aetiology

Study	Study type	Sample	Location	Cases vs. controls	MS patients
Bharanidharan 1997(1)	Survey	42 MS patients	Hungary		**Majority of patients born in Apr/Oct; Higher incidence of females born in Apr**
Salemi 2000(2)	Case-control	965 MS vs. Sicilian population	Sicily	No significant difference between cases and controls (p=0.25)	
Willer 2005(3)	Case-control	17,874 MS patients vs. all recorded births in Canada 1926-70	Canada	**Significantly fewer MS born in November vs. controls (p=0.0011)**; more MS born in May vs. controls (p=0.15)	
Willer 2005(3)	Case-control	11.502 incident MS cases vs. census controls	UK	**Significantly fewer MS born in Nov (p=0.009) and significantly more born in May (p<0.0001)**	
Willer 2005(3)	Pooled analysis (Canada, UK, Sweden, Denmark cohorts)	44,045 cases vs. controls	Canada, UK, Sweden, Denmark	**Significantly fewer MS born in Nov (p<0.0001) and more born in May (p<0.0001)**	

Sotgiu 2006(4)	Case-control	810 MS, 1069 unaffected siblings, 247,612 controls	Sardinia	**Significantly more MS births in spring vs. siblings (p=0.007-p=0.009) and general population (p=0.035-p=0.039)**	
Sadovnick 2007(5)	Case-control	11,465 RRMS vs. 11,553 unaffected siblings and all Canada births (1926-1970)	Canada	**Significantly fewer MS births in Nov vs. siblings (p=0.000039) and controls (p=0.000076); Significantly more MS births in May compared to population controls (p=0.043);** no significant difference in May births compared to sibling	
Sadovnick 2007(5)	Case-control	3,334 PPMS vs. 2,230 unaffected siblings and all Canada births (1926-1970)	Canada	No significant difference in month of birth between cases and siblings or population controls	
Fernandes de Abreu 2009(6)	Case-control	583 MS vs. both parents of each case	France	**Significantly fewer MS births in Nov (p=0.005);** non-significant excess of MS births in Apr; **Significantly fewer MS births in autumn (p=0.03)**	
Ramagopalan 2009(7)	Pooled case-control (Canada, Sweden, Norway)	4834 MS, 4056 controls, 659 unaffected siblings	Canada, Sweden, Norway	No significant difference between cases and controls	**HLA-DRB1*15-positive cases have significantly higher proportion of births in Apr (p=0.004) and lower in Nov (p=0.023) than HLA-DRB1*15-negative**
Bayes 2010(8)	Case-	1,309 MS vs. national and	Scotland	**Significantly more MS born in spring than expected (p<0.0001) and significantly fewer**	

	control	regional births		**born in autumn (p=0.01)**
Salzer 2010(9)	Case-control	9361 MS cases vs. all births in Sweden (1900-2007)	Sweden	**Significantly more MS births in June (p=0.004), significantly fewer MS births in Dec (p=0.042)**

Appendix 1A Table 2. UV exposure and MS aetiology

Study	Study type	Sample	Exposure	Cases vs. controls: crude	Cases vs. controls: adjusted
van der Mei 2003(10)	Case-control	136 cases, 272 age/sex-matched controls	Sun exposure 6-10yo, 11-15yo, 16-20yo		**Higher sun exposure 6-10 (p<0.01); 11-15yo (p=0.01);** **Higher sun exposure 6-15yo: OR: 0.31 (0.16, 0.59) (p<0.01)**
Islam 2007(11)	Twin case-control	79 disease/exposure discordant MZ twin pairs	Childhood UV-related exposures	**More time in sun during all seasons connotes less risk but only sig during spring (p=0.03); Suntanning (p=0.01) and beach activities (p=0.03) significantly protective**	
Dalmay 2010(12)	Matched case-control: Cuba, Martinique and Sicily	551 cases, 358 controls	Various UV-related behaviours/exposures prior to 15yo	**Protective:** **Shirt/shorts at leisure (p=0.0438); Shirtless/shorts in sun (p=0.0341); Outdoor sports (p=0.0044); Outdoor swimming (p=0.0021); Holiday at sea (p=0.0011); Water sports on holiday (p<0.0001); 1+ hr UV on weekend (p=0.0009); 1+hr UV on weekday (p=0.0001);** **Harmful: Hat/cap (p=0.0009); shirt under sun (p=0.0009); long-sleeve shirt (p=0.0446); Pants (p<0.0001); pants in sun (p=0.0002); Sunburn (p=0.0030)**	**Protective:** **1+hr UV on weekday (p=0.0105); 1+hr UV on weekend (p=0.0393); water sports on holiday (p<0.0001);** **Harmful: Pants in sun (p=0.0080)** **Dose-dependent UV:** **Weekday (p=0.04)** Weekend: (p>0.05)

Lucas 2011(13)	Case-control, Australia	216 with 395 age/sex-matched controls	Hours per day in sun in summer and winter; past, recent leisure sun exposure		Increasing sun in last 3yr inversely associated with MS risk in summer (AOR: 0.84 (95% CI: 0.72, 0.99)) and winter (AOR: 0.85 (95% CI: 0.72, 1.00)). Leisure UV 6yo to present inversely associated with MS risk (AOR: 0.70 (95% CI: 0.53, 0.94)).

Appendix 1A Table 3. Serum 25(OH)D: cases vs. controls

Study	Study type	Sample	Cases vs. controls	Per unit change	Categorical
Soilu-Hanninen 2005(14)	Case-control	40 cases, 40 controls	No difference in winter, but **significantly lower 25(OH)D in cases in summer (p<0.05)**		
Munger 2006(15)	Prospective nested case-control	257 cases vs. 514 matched controls		**Whites: OR: 0.59 (0.36, 0.97) per 50nmol/L increase;** No association among non-whites	**Whites: Trend: p=0.02;** No association among non-whites
Barnes 2007(16)	Case-control	29 cases vs. 22 age/sex-matched controls	No sig diff btw cases/controls (p=0.831)		
van der Mei 2007(17)	Case-control	136 MS, 272 age/sex-matched controls	No sig diff btw cases and controls		
Orton 2008(18)	Twin case-control	40 monozygotic, 59 dizygotic twin pairs	No sig association btw 25(OH)D and disease (OR: 1.24, p=0.43).		

Soilu-Hanninen 2008(19)	Nested case-control	23 MS, 23 age/sex/region-matched controls	No sig diff btw cases/controls (p=0.81)	
Correale 2009(20)	Case-control	58 RRMS, 40 PPMS, 60 matched controls	**Significantly lower 25(OH)D in RRMS vs. controls (p<0.00001)**; no significant difference between PPMS and controls	
Kragt 2009(21)	Case-control	103 cases, 110 controls	No sig diff btw cases/controls (Sum: p=0.269: Win: p=0.119))	**Women: Adj. OR: 0.75 (0.62, 0.91) per 10nmol/L increase winter 25(OH)D**; Adj. OR: 0.91 (0.80, 1.05) per 10nmol/L increase summer; No associations in men
Hølmoy 2009(22)	Case-control	36 RRMS, 20 OND, 18 ONIND	**Significantly higher median 25(OH)D in cases vs. OND (p=0.011) and ONIND (p=0.041)**	
Correale 2010(23)	Case-control	92 RRMS (58 remission, 34 relapse0, 30 controls	**Females: Significantly lower levels in MS vs. controls (p<0.0001); Males: Significantly lower 25(OH)D in cases vs. controls (RRRMS: p=0.0003; RREMS: p=0.01)**	
Shaygannejad 2010(24)	Case control	50 MS, 50 matched controls	**Significantly lower 25(OH)D in cases (48nmol/L) vs. controls (62nmol/L) p=0.036**	
Neau	Case-	170 MS, 170 matched controls	**25(OH)D significantly lower**	

2011(25)	control		**in cases vs. controls**	
Lucas 2011(13)	Case-control	216 cases, 395 age/sex-matched controls		**FDE risk decreased per 10nmol/L increase in 25(OH)D (AOR: 0.93 (95% CI: 0.86, 1.00)) and per 50nmol/L increase (AOR: 0.69 (95% CI: 0.48, 0.98)).**

Appendix 1A Table 4. Serum $1,25(OH)_2D$ and MS aetiology

Study	Study type	Sample	Cases vs. controls	Per unit change
Barnes 2007(16)	Case-control	29 cases vs. 22 age/sex-matched controls	No sig diff btw cases/controls (p=0.353)	
Correale 2009(20)	Case-control	58 RRMS, 40 PPMS, 60 matched controls	**Significantly lower 1,25(OH)2D in RRMS vs. controls (p<0.00003)**; no significant difference between PPMS and controls.	
Kragt 2009(21)	Case-control	103 cases, 110 controls	No sig diff btw cases/controls (Sum: p=0.830: Win: p=0.431))	No association btw 10-unit change in winter or summer 1,25(OH)2D in males or females
Correale 2010(23)	Case-control	92 RRMS (58 remission, 34 relapse0, 30 controls	**Females: Significantly lower levels in MS vs. controls (p=0.0006); Males: Significantly lower 25(OH)D in cases vs. controls (RRRMS: p=0.003; RREMS: p=0.002)**	

Appendix 1A Table 5. Serum 25(OH)D and clinical course

Study	Study type	Sample	Relapse/remission	MRI	Progression
Soilu-Hanninen 2005(14)	Case-control	40 cases, 40 controls	**Lower 25(OH)D during relapse vs. remission (p<0.05)**	No correlation btw 25(OH)D and gadolinium-lesions in brain/spinal cord	No correlation btw 25(OH)D and EDSS
van der Mei 2007(17)	Case-control	136 MS, 272 age/sex-matched controls			**Significant inverse correlation btw 25(OH)D and EDSS (p<0.0001)**
Soilu-Hanninen 2008(19)	Prospective cohort	23 cases, 23 controls	**Higher 25(OHD) during remission vs. relapse (p=0.012)**	No correlation btw 25(OH)D and MRI BOD or T2	Borderline non-sig inverse trend btw serum 25(OH)D and EDSS (p=0.065)
Smolders 2008(26)	Retrospective cohort	267 MS	**Significantly lower 25(OH)D for those having relapse (75.31) vs. not (97.63);** No association btw. 25(OH)D and relapse rate; **RR of no-relapse in previous 2-yrs increases 51% per 10nmol/L increase 25(OH)D (p=0.017)**		**Significantly lower levels in progressive vs. RRMS (p<0.001); Significant inverse association w/ EDSS (OR: -0.014 (-0.022, -0.006)**
Tremlett 2008(27)	Ecological	199 MS (142 RRMS)	Near-significant inverse correlation btw monthly relapse rate and deseasonalised 25(OH)D) (p=0.057)		
Correale 2009(20)	Case-control	58 RRMS, 40 PPMS, 60 matched controls	**Significantly lower 25(OH)D during relapse vs. remission (p<0.0001)**		
Kragt	Prospective	103 cases, 110			**Significant inverse correlation btw winter 25(OH)D and EDSS in**

2009(21)	cohort??	controls			**summer (p=0.044) and winter (p=0.020) in females;** no association in males
Hølmoy 2009(22)	Case-control	36 RRMS, 20 OND, 18 ONIND	No significant difference btw relapse/remission	No significant difference btw cases w/ Gadolinium-lesions and not	
Correale 2010(23)	Case-control	92 RRMS (58 remission, 34 relapse0, 30 controls	**Females: Significantly lower levels in relapse vs. remission (p<0.0001);** No significant difference in males (p=0.18)		
Simpson, Jr. 2010(28)	Prospective cohort	145 RRMS	**Significantly lower 25(OH)D in relapse vs. remission (p=0.006)**; **HR: 0.91 (0.85, 0.97) per 10nmol/L increase (basic analysis)**		
Weinstock-Guttman 2010(29)	Cross-sectional	193 CDMS		No significant association between 25(OH)D and MRI measures	**Significant inverse association between baseline 25(OH)D and baseline MSSS (p=0.029)**
Neau 2011(25)	Retrospective cohort	170 MS	No association between 25(OH)D & relapse rate in preceding year	No association between 25(OH)D and MRI measures within 12 months	**Significant inverse association between 25(OH)D and EDSS; 25(OH)D significantly lower in progressive vs. RRMS**

Appendix 1A Table 6. Serum $1,25(OH)_2D$ and clinical course

Study	Study type	Sample	Relapse/remission	MRI	Progression
Smolders 2008(26)	Cross-sectional	267 MS	**Significantly lower levels for those having relapse (120.52) vs. not (173.80);** No association btw. 1,25(OH)2D and relapse rate; No significant association btw 1,25(OH)2D and relapse in previous 2-yr (p=0.201)		**Significantly lower levels in progressive vs. RRMS (p=0.002);** No association with EDSS (p=1.00)
Correale 2009(20)	Case-control	58 RRMS, 40 PPMS, 60 matched controls	**Significantly lower 1,25(OH)2D during relapse vs. remission (p<0.00001)**		
Correale 2010(23)	Case-control	92 RRMS (58 remission, 34 relapse0, 30 controls	**Females: Significantly lower levels in relapse vs. remission (p<0.0001) ;** No significant difference in males (p=0.14)		
Weinstock-Guttman 2010(29)	Cross-sectional	193 CDMS		No significant relationship between 1,25(OH)2D and MRI measures	Inverse association between baseline 1,25(OH)2D and baseline MSSS (p=0.115)

Appendix 1A Table 7. Serum anti-HHV-6 IgG & MS onset and clinical course

Author	Cases	Assay	Type, period	MS Onset	Clinical course
Sola 1993(30)	126 MS, 500 controls	IFA (1:160 dil)	Case-control	**23% MS positive vs. 4% controls (p<0.0005)**	
Liedtke 1995(31)	36 MS, 27 neuro-AIDS, 24 controls	IFA	Case-control	14/36 MS cases positive, 5/24 controls (p=0.23)	
Nielsen 1997(32)	189 MS, 190 controls	Competitive ELISA	Case -control	100% cases positive, 188/190 controls positive. No difference in IgG titres	
Soldan 1997(33)	36 MS (22 RRMS, 14 CPMS), 31 OND, 21 OID, 14 control	EIA	Case-control	No difference between cases and controls	
Ablashi 1998(34)	16 MS, 8 OND, 72 controls	IFA	Case-control	15/16 MS (93.8%), 7/8 OND, 65/72 controls	
Enbom 1999(35)	55 MS, 20 controls	IFA	Case-control	Anti-HHV-6 IgG found in all cases and controls	
Ongradi 1999(36)	7 MS, 6 OND, 12 controls	ELISA	Case-control	**Higher titres in MS vs. controls**	
Albashi 2000(37)	21 MS, 20 OND, 25 controls	IFA	Case-control	19/21 MS (90.5%), 16/20 OND, 15/20 controls, **Higher titres in MS (1:320-1:2560) vs. controls (1:20-1:60)**	
Caselli 2002(38)	54 MS, 20 OND, 15 CIN, 82 controls	ELISA (anti-U92/*rep* only – HHV-6 latency gene product)	Case-control	47/54 MS (87%), 36/82 controls (p>0.05); 7/20 OND, 6/15 CIN **Significantly higher IgG titre in MS vs. controls (p<0.01)**	
Gutierrez 2002(39)	41 MS, 31 OND	ELISA	Case-control	**53.8% MS, 40% OND (p<0.05).** No difference in titre	
Gutierrez 2002(39)	27 MS, 31 OND	ELISA	Case-control (cases followed	**100% MS, 40% OND (p<0.005)** No difference in titre	

			prospectively)		
Beck 2003(40)	27 MS	IFA	Cross-sectional	19/27 MS	
Chapenko 2003(41)	26 MS, 21 OND, 150 controls	IFA	Case-control		Elevated HHV-6 IgG in 4/6 exacerbation samples negative for HHV-6 IgM and viral load
Villoslada 2003(42)	98 MS (49 RRMS, 49 SPMS), 53 CIS, 50 controls	ELISA	Nested case-control	No difference in frequency or titre of IgG between cases/controls	
Sundstrom 2004(43)	73 MS cases, prospective, 219 controls	ELISA	Prospective cohort, case-control	All samples positive; **Significantly higher titres in cases vs. controls ($p<0.05$). Significantly higher titres when samples collected <5yr prior to Sx onset**	
Sundstrom 2004(43)	161 MS cases, retrospective, 483 controls	ELISA	Retrospective cohort, case-control	All cases positive. 481/483 controls positive	
Derfuss 2005(44)	38 MS (28 RRMS, 10 CPMS), 13 possible MS, 21 OIND, 5 OND	ELISA	Case-control	23/28 RRMS, 9/10 CPMS, 13/13 possible MS, 19/21 OIND, 4/5 OND	
Virtanen 2007(45)	27 RRMS, 19 CPMS, 27 OND	IFA	Case-control	**27/27 RRMS, 19/19 CPMS, 18/27 OND ($p=0.001$ RRMS vs. control; $p=0.007$ CPMS vs. control)**	
Kuuisto 2008(46)	17 MS, 17 non-MS twins	ELISA	Case-control	15/17 MS (88%), 12/14 controls	
Comabella 2010(47)	25 MS and 46 full siblings	ELISA	Case-control	92% cases, 93% siblings	
Behzad-Behbahani 2011(48)	22 RRMS, 7 SPMS, 1 PPMS, 20 HBD	EIA	Case-control/ 6-month Prosp cohort	100% MS vs. 75% controls	No difference by MS type
Khaki 2011(49)	61 MS, 60 controls	ELISA, IFA	Case-control	Significantly higher frequency of detection in cases ($p=0.04$)	

Appendix 1A Table 8. Serum anti-EBV IgG and MS onset and clinical course

Author	Cases	Assay & antigen	Type, period	MS Onset	Clinical course
Sumaya 1980(50)	157 MS, 81 controls	IFA: EBV-VCA	Case-control	**155/157 MS, 76/81 controls; IgG titre significantly higher in patients than controls ($p<0.05$)**	
Bray 1983(51)	313 MS, 406 age/sex-matched controls & OND	IFA: EBV-VCA	Case-control	**309/313 MS, 363/406 controls ($p<0.05$)**	
Larsen 1985(52)	93 MS, 93 age/sex-matched controls	IFA: EBV-VCA, EBV-EBNA	Case-control	**93/93 MS, 78/93 controls ($p<0.0001$). IgG titre significantly higher in patients vs. controls ($p<0.0001$).**	
Sumaya 1985(53)	104 MS, 104 age/sex-matched controls	IFA: EBV-VCA, EBV-EBNA	Case-control	**104/104 MS, 99/104 controls ($p<0.05$)**	
Shirodaria 1987(54)	26 MS, 26 controls	IFA: EBV-VCA	Case-control	**26/26 MS, 24/26 controls ($p<0.05$)**	
Ferrante 1987(55)	30 MS, 51 residency-matched OND	IFA: EBV-VCA	Case-control	**29/30 MS, 31/51 controls**	
Munch 1997(56)	138 MS, 138 age/sex-matched controls	ELISA: EBV-EBNA, EBV-EA	Case-control	**137/138 MS, 124/138 controls**	
Enbom 1999(35)	55 MS, 20 OND	IFA	Case-control	No difference in serum EBV IgG between cases/controls	
Wandinger 2000(57)	108 MS, 163 controls	ELISA, EBV-EA, EBV-EBNA, EBV-VCA	Case-control	**EBV-EBNA: 100% cases, 90.1% of controls ($p=0.002$)** No significant difference in other EBV antigens.	
Ascherio 2001(58)	18 female MS cases w/ blood collected prior to Sx onset, 36 age-matched female controls	IFA: EBV-EBNA, EBV-VCA, EBV-EA	Nested case-control	**Significantly greater EBNA-1 titre for cases vs. controls ($p=0.02$); EBNA-2 ($p=0.01$).**	
Ascherio 2001(58)	126 female MS cases w/ blood collected after MS onset, 252 age-matched female controls	IFA: EBV-EBNA, EBV-VCA, EBV-EA	Nested case-control	**Significantly greater EBNA-1 titre for cases vs. controls ($p<0.001$); EBNA-2 ($p<0.001$), EBNA ($p<0.001$), VCA ($p<0.001$).**	

Villoslada 2003(42)	49 RRMS, 50 SPMS, 53 CIS, 50 controls	ELISA, EBV-EA, EBV-EBNA	Case-control	**Higher EBNA titres in RRMS (p=0.041) and CIS (p<0.0001) vs. controls** No difference in proportions with detectable EBNA.	**EBNA titres correlate with earlier stage of disease (shorter duration (p<0.0001), lower age at onset (p<0.0001), lower EDSS (p<0.0001)**
Haahr 2004(59)	53 MS, 53 age/sex/social milieu-matched controls	ELISA: EBV-EBNA	Case-control	**100% cases positive vs. 94% controls**	
Sundstrom 2004(43)	73 MS cases, prospective, 219 controls	ELISA: EBV-VCA, EBV-EBNA	Prospective cohort, case-control	73/73 cases positive for EBNA and VCA vs. 210/219 for EBNA and 217/219 for VCA. **Significantly higher EBNA titres in cases**; VCA titres not significantly different. **Restricted to samples <5yr prior to Sx onset, significantly higher EBNA titres in cases, significantly lower titres in cases.**	
Sundstrom 2004(43)	161 MS cases, retrospective, 483 controls	ELISA: EBV-VCA, EBV-EBNA	Retrospective cohort, case-control	**160/161 cases positive for EBNA vs. 459/483 controls.** 161/161 cases positive for VCA vs. 473/483 controls	
Ponsonby 2005(60)	136 MS, 272 age/sex-matched controls	ELISA: EBV-EBNA, EBV-VCA	Case-control	**100% cases and 97% controls positive for EBV-VCA IgG. Cases had higher composite EBV IgG titres (p<0.001).**	
Zivadinov 2006(61)	140 MS, 131 age/sex-matched controls	ELISA: EBV-VCA	Case-control	126/133 MS, 131/131 controls	
Riverol 2007(62)	172 MS, 85 age/sex-matched controls	ELISA, EBV-EBNA, EBV-EA	Case-control	**Greater proportion of positive EBNA in MS vs. control (p<0.05) and higher titres vs. controls (p<0.05)**	
Myhr 1998(63)	144 MS, 170 age/sex/area-matched controls	ELISA	Case-control	**144/144 MS, 162/170 controls (p=0.008)** **Greater frequency of detection of EBV-VCA (p<0.000001), EBV-EBNA (p=0.01).**	
DeLorenze 2006(64)	42 MS (collected prior to onset), 79 age/sex/collection-date-matched controls	IFA, EBV-EA, EBV-EBNA, EBV-VCA	Nested case control from prospective cohort	All cases/controls positive for EBV IgG **Significantly higher titres of anti-EBNA IgG in cases vs. controls (p=0.02).** No significant difference in EBV VCA	
Buljevac	54 RRMS	ELISA – EBV-EA,	Prospective	EBV-VCA positive in all subjects; EBV-EBNA positive in	

2005(65)		EBV-EBNA, EBV-VCA	cohort (mean 1.7yr)	51/54 subjects	
Farrell 2009(66)	50 MS (25 RRMS, 25 PPMS), 50 CIS	Quantitative chemoluminscent assay: EBV-VCA, EBV-EBNA,	5-yr prospective	**100% samples positive to EBV-VCA IgG.** RRMS significantly higher EBNA titres than PPMS or CIS ($p<0.001$).	**Subjects with activity on gadolinium-MRI had higher mean EBNA IgG ($p<0.001$). EBNA IgG titre positively correlated with # T2 lesions ($p<0.001$). EBNA IgG titre significant predictor of increase in T2 lesion volume ($p=0.025$).** No association between VCA IgG and MRI measures.
Khaki 2011(49)	61 MS, 60 controls	ELISA, IFA	Case-control	No difference between cases and controls	

Appendix 1A Table 9. Serological marker of HHV-6 reactivation (anti-HHV-6 IgM) and MS onset and clinical course

Author	Cases	Assay	Type, period	MS Onset	Clinical course
Liedtke 1995(31)	36 MS, 27 neuro-AIDS, 24 controls	IFA	Case-control	1/36 MS cases positive, 0/24 controls positive	
Soldan 1997(33)	36 MS (22 RRMS, 14 CPMS), 31 OND, 21 OID, 14 control	EIA	Case-control	**Significantly more RRMS than controls (p<0.0011),** borderline significantly more CPMS than controls (p=0.014)	
Ablashi 1998(34)	16 MS, 8 OND, 72 controls	IFA	Case-control	**9/16 MS, 1/8 OND, 4/21 MS**	
Enbom 1999(35)	55 MS, 20 controls	IFA	Case-control	1/55 cases positive, controls not tested	
Friedman 1999(67)	25 MS, 14 other autoimmune, 19 controls	IFA		21/25 MS, 3/19 controls (p=0.0001)	
Ongradi 1999(36)	7 MS, 6 OND, 12 controls	ELISA	Case-control	**Higher titres in MS vs. controls**	
Albashi 2000(37)	21 MS, 20 OND, 25 controls	IFA	Case-control	**15/21 MS, 4/20 OND, 3/20 controls**	
Taus 2000(68)	23 RRMS, 8 controls	IFA	Case-control	16/23 MS positive – similar to local population	
Gutierrez 2002(39)	41 MS, 31 OND	ELISA	Case-control	No samples positive for HHV-6 IgM	
Gutierrez 2002(39)	27 MS, 31 OND	ELISA	Case-control (cases followed prospectively)	13/27 cases positive for HHV-6 IgM	
Beck 2003(40)	27 MS	IFA	Cross-sectional	0 MS	
Chapenko 2003(41)	26 MS, 21 OND, 150 controls	IFA	Case-control		2/6 exacerbation with no detectable viral load
Villoslada 2003(42)	98 MS (49 RRMS, 49 SPMS), 53 CIS, 50 controls	ELISA	Nested case-control	**RRMS & CIS have higher IgM titre than control (p<0.0001).**	**IgM positively correlated w/ shorter disdur and lower EDSS**

Riverol 2007(62)	172 MS, 22 CIS, 85 control	ELISA	Case-control	35.1% MS, 50% CIS, 34% controls No difference in IgM titres
Kuuisto 2008(46)	17 MS, 17 non-MS twins	ELISA	Case-control	1 MS, 0 controls
Khaki 2011(49)	61 MS, 60 controls	ELISA, IFA	Case-control	Significantly higher frequency of IgM in cases (p=0.001)

Appendix 1A Table 10. Serological marker of EBV reactivation (anti-EBV-EA IgG) and MS onset & clinical course

Author	Cases	Assay & antigen	Type, period	MS Onset	Clinical course
Wandinger 2000(57)	108 MS, 163 controls	ELISA, EBV-EA, EBV-EBNA, EBV-VCA	Case-control	No difference in EBV reactivation between cases (13.9%) and controls (11.2%)	
Wandinger 2000(57)	19 MS	ELISA, EBV-EA, EBV-EBNA, EBV-VCA	1-yr prospective		**Serological evidence of EBV reactivation more frequency during exacerbation (72.7%) vs. none of controls**
Ascherio 2001(58)	18 female MS cases w/ blood collected prior to Sx onset, 36 age-matched female controls	IFA: EBV-EBNA, EBV-VCA, EBV-EA	Nested case-control	**Significantly greater EA-D (p=0.04).**	
Ascherio 2001(58)	126 female MS cases w/ blood collected after MS onset, 252 age-matched female controls	IFA: EBV-EBNA, EBV-VCA, EBV-EA	Nested case-control	**Significantly greater EA-D (p=0.03), EA-R 9p=0.03),**	
Villoslada 2003(42)	49 RRMS, 50 SPMS, 53 CIS, 50 controls	ELISA, EBV-EA, EBV-EBNA	Case-control	No difference in EBV-EA	

Riverol 2007(62)	172 MS, 85 age/sex-matched controls	ELISA, EBV-EBNA, EBV-EA	Case-control	No difference in EBV-EA	
Myhr 1998(63)	144 MS, 170 age/sex/area-matched controls	ELISA	Case-control	**Greater frequency of detection of EBV-EA (p<0.0001).**	
DeLorenze 2006(64)	42 MS (collected prior to onset), 79 age/sex/collection-date-matched controls	IFA, EBV-EA, EBV-EBNA, EBV-VCA	Nested case control from prospective cohort	No significant difference in EBV-EA	
Buljevac 2005(65)	54 RRMS	ELISA – EBV-EA, EBV-EBNA, EBV-VCA	Prospective cohort (mean 1.7yr)	EBV-EA positive in 26/54 subjects	
Buljevac 2005(65)	54 RRMS, 52 age-matched controls	ELISA – EBV-EA	Nested case-control	**26/54 MS vs. 13/52 controls (p=0.013) and significantly higher titres in MS (p<0.001)**	No difference in EBV IgG detection or titre between relapse/remission samples.

Appendix 1A Table 11. Serum HHV-6 viral load and MS onset and clinical course

Author	Cases	Assay	Type, period	MS Onset	Clinical course
Wilborn 1994(69)	21 MS, 28 OND, 57 controls	Nested-PCR	Case-control	No samples positive for HHV-6 DNA	
Martin 1997(70)	26 MS, 39 controls	PCR	Case-control	0% cases and controls positive	
Soldan 1997(33)	50 MS, 19 OND, 10 OID, 18 controls	Nested-PCR	Case-control	**15/50 MS, 0/47 non-MS (p<0.0001)**	
Fillet 1998(71)	32 MS, 21 OND, 13 controls	PCR	Case-control	2/32 MS, 1/21 OND, 0/13 controls	
Mirandola 1999(72)	32 RRMS, 12 OND	Nested-PCR	Case-control	0% cases and controls positive	
Goldberg 1999(73)	24 MS, 16 OND, 14 controls	Nested-PCR	Case-control w/ longitudinal sampling of cases	1/24 MS, 0/16 OND, 0/14 controls	No significant difference in detection in relapse (25) vs. remission (34) vs. progressive 23
Akhyani 2000(74)	38 MS, 19 controls	Nested-PCR	Case-control	8/34 MS, 0/19 controls (p=0.02)	
Hay 2000(75)	29 MS, 7 controls	Nested PCR	Case-control	2/29 (7%) MS samples, 1/7 (14%) controls	
Tomsone 2001(76)	56 MS, 21 CNS NMD, 150 HBD	PCR	Case-control	**61.9% MS positive vs. 28.6% NMD and 28.7% HBD. (p<0.01)**	
Alvarez-Lafuente 2002(77)	103 RRMS, 46 controls	Quant-PCR	Case-control	**14.6% MS positive, 0/46 controls (p<0.001)**	No sig difference between relapse (12.1%)/remission (15.7%)
Berti 2002(78)	59 MS, 70 controls	Nested PCR	5-month prospective	**23% case samples positive, 0% controls (p<0.0001)**	**22% samples during relapse positive, 5.6% during remission (p=0.008)**
Gutierrez 2002(39)	41 MS, 31 OND	PCR	Case-control	No HHV-6 DNA detected in any sample	

Gutierrez 2002(39)	27 MS	PCR	Prospective	No HHV-6 DNA detected in any sample	
Tejada-Simon 2002(79)	33 MS, 21 controls	PCR	Case-control	22/33 MS, 7/21 controls	
mmari 2003(80)	24 MS, 13 OND, 20 controls	Nested-PCR	Case-control	0 MS, 0 OND, 1/20 controls	
Beck 2003(40)	27 MS	Nested-PCR	Cross-sectional	0 MS	
Chapenko 2003(41)	26 MS, 21 OND, 150 controls	Nested-PCR	Case-control	**8/16 MS, 0/21 OND, 0/150 controls**	**8/13 relapse, 0/6 remission**
Alvarez-Lafuente 2004(81)	105 RRMS. 49 controls	Quant-PCR	Case-control	**17/105 RRMS, 0/49 controls (p=0.003)** No significant difference in viral load between cases/controls (p=0.06)	**Significantly higher viral load in relapse vs. remission (p=0.04)**
Alvarez-Lafuente 2004(82)	189 RRMS (84 Tx w/ IFN-β, 105 untreated), 65 controls	Quant-PCR	Cross sectional (cases from prosp. study)	34/189 MS positive, 0/65 controls	No significant difference in proportion positive during relapse/remission (p=0.45) **Viral load significantly higher during relapse in untreated than treated (p=0.001).**
Hollsberg 2005(83)	33 MS, 18 controls	PCR	Cross sectional (cases from prosp. study)	25% MS cases, 39% controls	**Significantly greater frequency of HHV-6 DNA detection in patients with higher disease activity (p=0.023)**
Alvarez-Lafuente 2006(84)	57 RRMS, 57 controls	Quant-PCR	12-month prospective	**80.7% RRMS samples positive vs. 29.8% controls (p<0.001)**	**19.3% positive during relapse vs. 1.9% in remission (p=0.028)**
Alvarez-Lafuente 2007(85)	53 RRMS, 53 SPMS, 106 HBD	QRT-PCR	Cross-sectional	**53.8% RRMS vs. 30.1% HBD (p=0.001);** 31.6 SPMS vs. 21.9% HBD (p=0.16).	**53.8% RRMS vs. 31.6% SPMS (p=0.027);** **76.5% relapse RRMS vs. 48.7% remission RRMS (p=0.037)**
Riverol 2007(62)	172 MS, 22 CIS, 85 control	PCR	Case-control	No HHV-6 DNA found in any sample	
Virtanen 2007(45)	27 RRMS, 19 CPMS, 27 OND	Multiplex PCR	Case-control	0 MS, 0 OND	
Kuuisto 2008(46)	17 MS, 17 non-MS twins	PCR	Case-control	0 MS, 0 controls	

Ahram 2009(86)	36 MS, 3 OND, 34 controls	Nested-PCR	Case-controls	8/34 MS positive, 8/37 non-MS positive	
Alvarez-Lafuente 2009(87)	103 RRMS, 103 controls	Quant-PCR	Case-control	**24/103 MS, 0/103 controls (p<0.0001)**	
Franciotta 2009(88)	54 RRMS, 10 OND, 15 HC	Real-time PCR	Case-control	0% cases and controls with detectable HHV-6 DNA	
Behzad-Behbahani 2011(48)	22 RRMS, 7 SPMS, 1 PPMS, 20 HBD	Nested PCR	Case-control/ 6-month Prosp cohort	**33% MS vs. 5% controls (p=0.001)**	9/22 RRMS vs. 1/7 SPMS and 0/1 PPMS positive for DNA (p=0.36).
Garcia-Montojo 2011(89)	54 MS,	QPCR	24 month prosp cohort		Detectable HHV-6 DNA associated with more severe relapse (p=0.01); HHV-6 DNA more frequent in relapse vs. remission samples (p=0.0002). No assoc

Appendix 1A Table 12. Serum EBV viral load and MS onset and clinical course

Author	Cases	Assay	Type, period	MS Onset	Clinical course
Marin 1997(70)	26 MS, 39 OND	Nested-PCR	Case-control	No viral DNA in any sample	
Wandinger 2000(57)	19 MS	PCR	1-yr prospective		**Evidence of EBV reactivation more frequency during exacerbation (72.7%) vs. none of controls**
Alvarez-Lafente 2004(81)	105 RRMS, 49 controls	Quant-PCR	Case-control	No significant difference in proportions positive for EBV DNA, nor in EBV viral load (p=0.08)	No difference in EBV viral load in relapse/remission
Wagner 2004(90)	31 MS (18 collected before Sx onset, 13 before Dx), 62 age/sample-date-matched controls	PCR	Nested case-control	EBV DNA present in 9/31 MS vs. 10/62 controls (p=0.12)	
Buljevac 2005(65)	51 exacerbation samples from 48 RRMS	PCR	Prospective cohort (mean 1.7yr)		3/51 exacerbation samples positive, none of 17 remission samples positive
Hollsberg 2005(83)	33 RRMS, 18 controls	PCR	Case-control	Similar detection in cases (17%) and controls (6%)	**Significantly greater frequency of EBV DNA detection in patients with higher disease activity (p=0.018)**
Alvarez-Lafuente 2006(84)	57 RRMS, 57 controls	Quant-PCR	Case-control	No difference in detection of EBV DNA (p=0.475)	No difference in EBV DNA between relapse/remission (p=0.615)
Riverol 2007(62)	172 MS, 85 age/sex-matched controls	PCR	Case-control	No viral DNA in any sample	
Virtanen 2007(45)	26 MS, 19 possible MS, 26 OND	PCR	Case-control	No viral DNA in any sample	
Farrell 2009(66)	50 MS, 50 CIS	PCR	5-yr prospective	No subjects with evidence of EBV reactivation	
Franciotta	54 RRMS, 10	Real-time	Case-control	0% cases and controls with detectable EBV DNA	

2009(88)	OND, 15 HC	PCR		
Garcia-Montojo 2011(89)	54 MS,	QPCR	24 month prosp cohort	No association between EBV viral load and occurrence of or severity of relapse

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Chapter 2. Trends in the epidemiology of multiple sclerosis in Greater Hobart, Tasmania: 1951 to 2009

2.1 Preface

The previous chapters have described MS as it is at the global level. However MS is an individual and local disease, and the distribution of MS in a community can evolve markedly over time. While the study of high-prevalence areas can be of significant importance for scientific research and hypothesis generation, it is the data from local studies that policymakers draw upon to allocate resources and more particularly, make plans for the allocations which might be required in the future. The last study to systematically evaluate MS epidemiology in the Greater Hobart area was completed in 1981. Thus it was decided to undertake a follow-up study to evaluate MS epidemiology in the area more recently, and to ascertain how its distribution has changed over time.

This chapter will investigate the epidemiology of MS in the Greater Hobart region in 2001 and 2009, including prevalence in each year, and the mean annual incidence and mortality rates over that interval. In combination with the preceding studies of MS epidemiology in Greater Hobart, this chapter will evaluate the change in MS prevalence between 1961 and 2009, the change in the mean annual incidence rate between 1951-61 and 2001-9, the change in the mean annual morality rate between 1951-9 and 2001-9, and changes in the distribution of these epidemiological measures by age, sex and ancestry, yielding a comprehensive analysis of the evolution of MS epidemiology in a high-prevalence location over a span of 58 years. This chapter was published in Journal of Neurology, Neurosurgery & Psychiatry (Appendix 2A). The discussion notes (grey boxes) are added for this thesis and were not part of the original publication.

2.2 Introduction

Multiple sclerosis (MS) is a disease of complex aetiology, including genetic and environmental elements.(1-3) A key part of unraveling MS aetiology comes from studies investigating its occurrence and distribution within communities and globally. Time-trend studies, where serial cross-sectional studies are analyzed over time, describe the evolution of MS in populations. These studies allow hypothesis generation about MS aetiology and provide invaluable data for the resource allocation and care for those affected.

Australia has played an important role in MS research, with a number of epidemiological studies undertaken.(4-8) Two landmark studies provided important data concerning the geographic and temporal distribution of MS: the first, by McCall and colleagues(4) in 1968, and the second by Hammond and colleagues(5) in 1988, investigated the epidemiology of MS in the cities of Newcastle (NSW), Perth (WA), and Hobart (TAS). Both studies demonstrated a significant latitudinal gradient characterized by higher prevalence and incidence rates with increasing latitude: the MS prevalence and incidence in Hobart (42.8°S) were nearly double that of Newcastle (32.9°S) and Perth (31.6°S). Indeed, other work has demonstrated that the MS prevalence in Hobart is over 6-times that of northern Queensland.(6) This latitudinal gradient has also been observed in New Zealand(9), Japan(10), France(11) and the USA(12) and has been borne out in meta-analyses of prevalence(13) and incidence.(14) Additionally, both prevalence and incidence increased between the McCall(4) and Hammond(5) studies in each of the three study sites. This temporal gradient continued in 1996, when Barnett and colleagues[10] undertook a follow-up study in Newcastle in 1997.

While Newcastle has been the focus of continued follow-up in the three-city studies, there have been no follow-up studies in Hobart. We have thus undertaken a two-stage survey of MS frequency in Hobart in 2001 and 2009 and here present the results of a time-trend analysis of MS epidemiology in Hobart over the 58-year period from 1951 to 2009.

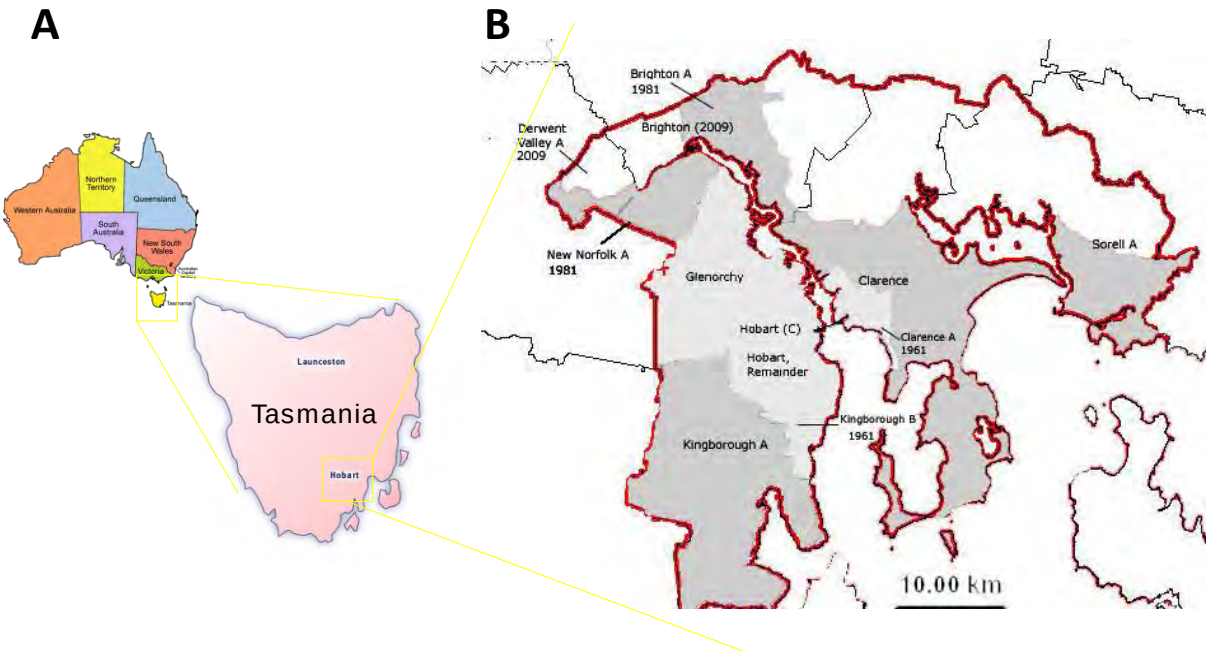
2.3 Methods

2.3.1 Study region and population

The Greater Hobart Statistical Division, hereafter referred to as Hobart, lies astride the lower Derwent River in the island state of Tasmania, at latitude 42.8°S. Since 1961, the statistical division has grown from 272 km^2 to its present size of 1,360 km^2 (Figure 2.1).

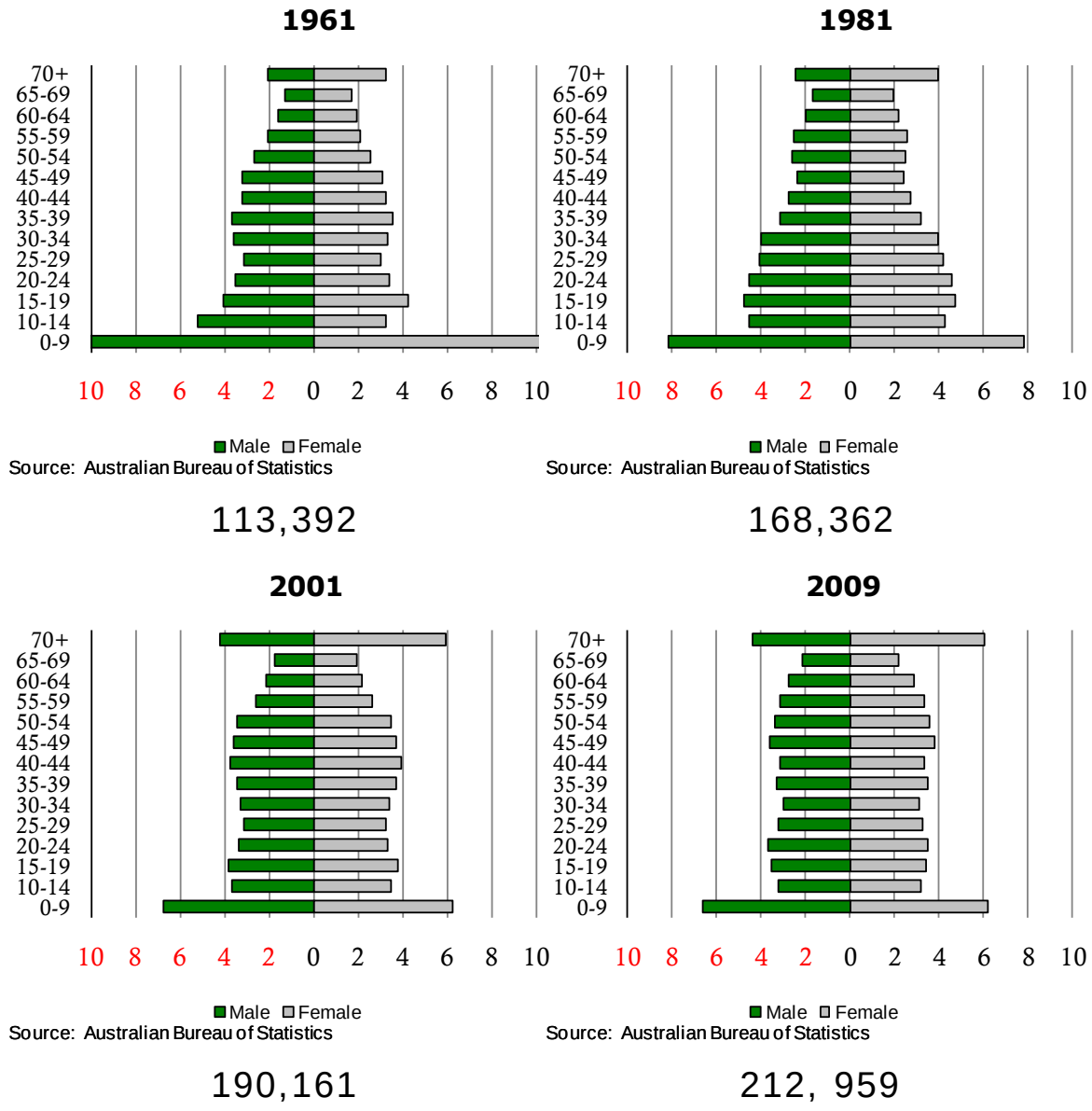
Figure 2.1. A. Location of Tasmania within Australia and Greater Hobart within Tasmania. B. Growth of the Greater Hobart Statistical Division (SD) over 1961-2009.

Light shaded areas show the boundaries in 1961; darker shaded areas show the boundaries in 1981; thick lines demarcate current boundaries. Alternate names for statistical localized areas in 1961 or 1981 vs. present are denoted by the appropriate year.



Despite the 400% increase in area, the population increased only 86.9% between 1961 and 2009, from 113,932 to 212,959. The population-structure changed appreciably however, from a "youth-bulge" distribution in 1961 and 1981, to an "apple-core" distribution in 2001 and 2009, reflecting an older age-structure (Figure 2.2).

Figure 2.2. Change in age/sex-distribution and populations of Greater Hobart, 1961-2009.



2.3.2 Context and case ascertainment

The 2001 prevalence study was done as part of the MS Longitudinal Study, with prevalence day on 7 August 2001. The 2009 prevalence study was done as part of the MS Prevalence and Genetics Study, with prevalence day on 1 January 2009. Both studies were approved by the Southern Tasmania Health and Medical Research Ethics Committee.

Cases for both prevalence studies were identified by direct neurological referral or vicariously via the local MS Society or participation in other MS studies conducted by the Menzies Research Institute. All cases were diagnosed by neurologist prior to inclusion in either prevalence study.

Data on prevalence in 1961 and 1981, and on incidence and mortality during 1951-61 and 1971-81 were extracted from the McCall(4) and Hammond(5) publications.

Only cases satisfying the requirements for definite MS by the 2001 McDonald(15) criteria in 2001 and the 2005 McDonald(16) criteria in 2009 were included. In the McCall study, the Allison and Millar criteria(17) were used, including probable, early-probable and possible cases. In the Hammond study, the Rose(18) criteria were used, including definite, probable and possible cases.

2.3.3 Epidemiological measures

2.3.1 Divisions of epidemiological measures

There are two major divisions of prevalence, incidence and mortality used in this analysis: by sex and by birthplace. Sex is divided into male and female, with the value for all persons referred to as the total. Birthplace is divided into Australian-born, which includes those persons born in any of the states of Australia, and overseas-born, which includes those persons born outside the territorial borders of Australia; the value for all persons, regardless of birthplace, is referred to as the aggregate.

2.3.2 Calculation of epidemiological measures

Crude point prevalence was calculated by dividing the number of cases in the study region on prevalence day by the population on prevalence day, expressed as a proportion per 100,000.

Crude mean annual incidence rate (incidence) was calculated by dividing the number of new cases with symptom-onset and living in the study region during the observation period by an estimate of the person-years of observation on the population at risk. For comparability across studies, total person-years were calculated as the mid-point population multiplied by the length in years of the observation

period. Incidence was expressed as a rate per 100,000 person-years. The crude mean annual mortality rate (mortality) was calculated in an analogous manner.

To calculate prevalence by birthplace for 2001 and incidence by birthplace for the period 2001-09, populations by birthplace from the 2001 and 2006 censuses were used, respectively. To calculate the 2009 prevalence by birthplace, we assumed that the proportions of overseas-born and Australian-born in each 5-year age group in 2009 were identical to those in the 2006 Census.

2.3.3.3 Calculation of distributions by birthplace

For the McCall and Hammond studies, prevalence and incidence were not reported with stratification by birthplace and by sex. Using the distribution by sex and by birthplace provided in each publication, we were able to calculate the distribution by birthplace and by sex of prevalence for each study and incidence for the Hammond study.

Methods note 2.1: Estimation of sex and birthplace-specific prevalence and incidence from 1988 Hammond study

Incidence and prevalence data from the 1988 study of Hammond and colleagues were not reported with stratification by both birthplace and by sex. It was necessary to estimate the missing data from the information provided in the published papers and population estimates provided by the Australian Bureau of Statistics. Methods note 2.1 Table A presents the available data as reported by Hammond and colleagues, along with population data from the Australian Bureau of Statistics.

Methods note 2.1 Table A. Sex and birthplace-specific information provided by Hammond and colleagues for MS prevalence, and population data from Australian Bureau of Statistics

	Australian-born			Overseas-born			Aggregate		
	Counts	Population	Prevalence	Counts	Population	Prevalence	Counts	Population	Prevalence
Males		71,466			11,139		43	82,720	52.0
Females		75,639			10,048		82	85,641	95.8
Total	94	147,105	63.9	31	21,187	146.4	125	168,363	74.2

It is not possible to uniquely determine the missing data on counts (number of cases) stratified by sex and birthplace, but it is possible to make reasonable estimates of these quantities as below:

Firstly, it was possible to calculate the overall prevalence sex ratio from these data as:

$$PSR = \frac{p_f}{p_m} = \frac{c_f / Pop_f}{c_m / Pop_m} = \frac{82/85641}{43/82720} = 1.84$$

where p_m and p_f denote the estimated prevalence of males and females respectively, c_m and c_f denote the respective counts (number of cases), and Pop_m and Pop_f denote the respective population estimates.

Note also that the overall prevalence is a population-weighted average of the each birthplace-specific prevalence estimate. For females, for example:

$$p = \left(\frac{Pop_f{}^A + Pop_m{}^A}{Pop_f + Pop_m} \right) \times p^A + \left(\frac{Pop_f{}^O + Pop_m{}^O}{Pop_f + Pop_m} \right) \times p^O$$

where p, p^A and p^O denote prevalence estimates and the superscript A denotes Australian-born and the superscript O denotes overseas-born.

I assumed that the overall prevalence sex ratio is approximately equal to a population-weighted average of the each birthplace-specific prevalence sex ratio. Specifically, I assumed that:

$$\frac{p_f}{p_m} \approx \left(\frac{Pop_f{}^A + Pop_m{}^A}{Pop_f + Pop_m} \right) \times \left(\frac{p_f{}^A}{p_m{}^A} \right) + \left(\frac{Pop_f{}^O + Pop_m{}^O}{Pop_f + Pop_m} \right) \times \left(\frac{p_f{}^O}{p_m{}^O} \right)$$

To estimate the missing prevalence data, I stipulated the number of Australian-born male cases $c_m{}^A$, calculated the remaining counts using the marginal relations $c_m{}^A + c_m{}^O = 43$, $c_m{}^A + c_f{}^A = 94$ and $c_f{}^A + c_f{}^O = 82$ taken from the row and column totals of the table, and calculated the approximate prevalence sex ratio using the formula given previously. I then varied the value stipulated for $c_m{}^A$ until

the approximate prevalence sex ratio was closest to the calculated prevalence sex ratio of $PSR = 1.84$. The count that provided the closest approximate value was ${c_m}^A = 32$.

The assumed relation between the overall prevalence sex ratio p_f / p_m and the birthplace-specific prevalence sex ratios ${p_f}^A / {p_m}^A$ and ${p_f}^O / {p_m}^O$ will provide a reasonable approximation provided that the birthplace-specific ratio are not too different in magnitude. Suggesting that they might not be different in magnitude, the mean prevalence sex ratio from 9 studies conducted in Australasia between 1970 and 1990 was 2.42 whereas the corresponding mean from 6 studies conducted in the United Kingdom during the same period was 1.90. Given that Hammond and colleagues reported that 71% of overseas-born patients with MS living in Hobart were born in the United Kingdom and Ireland, it is unlikely that there would have been a difference in the prevalence sex ratios of sufficient magnitude to invalidate the results of this study.

This procedure was also applied to estimate sex and birthplace-specific incidence for 1981, allowing the assessment of the change in sex ratio over time.

2.3.4 Age-standardisation

All age-standardisation was done using the direct method.(19)

No single population could be used for age-standardisation in this analysis given the variability in the presentation of age-specific rates in the McCall and Hammond publications. Total prevalence and prevalence by birthplace were age-standardised to the 1961 Hobart population. This was necessary because neither the McCall nor the Hammond studies provided age-specific prevalence by birthplace. To allow comparisons with 1961, we standardised prevalence in 2001 and 2009 to the 1961 population. The prevalence data reported for the Hammond study are standardised to the 1981 Australian

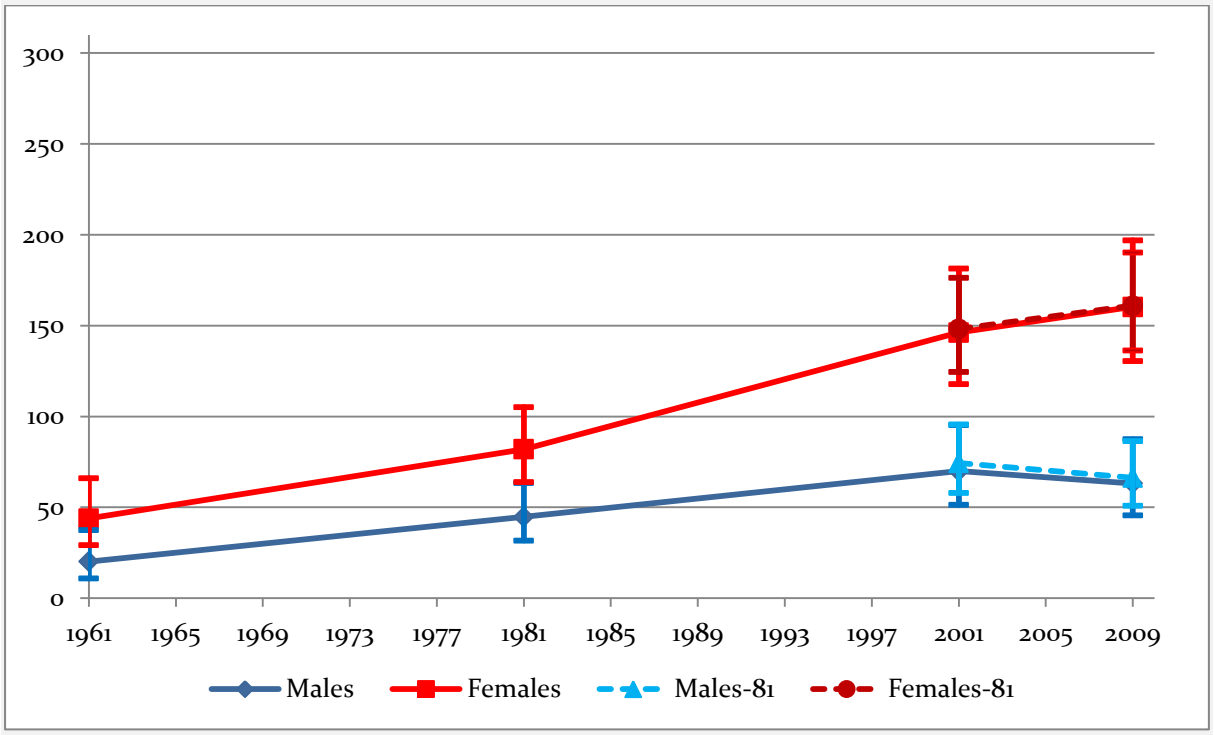
population and are not strictly comparable - to assess possible confounding bias, we performed a sensitivity analysis over the time points 1981, 2001 and 2009 by standardising the 2001 and 2009 prevalence data to the 1981 Australian population.

Methods note 2.2. Differential age-standardisation of crude 1981 prevalence by sex and birthplace

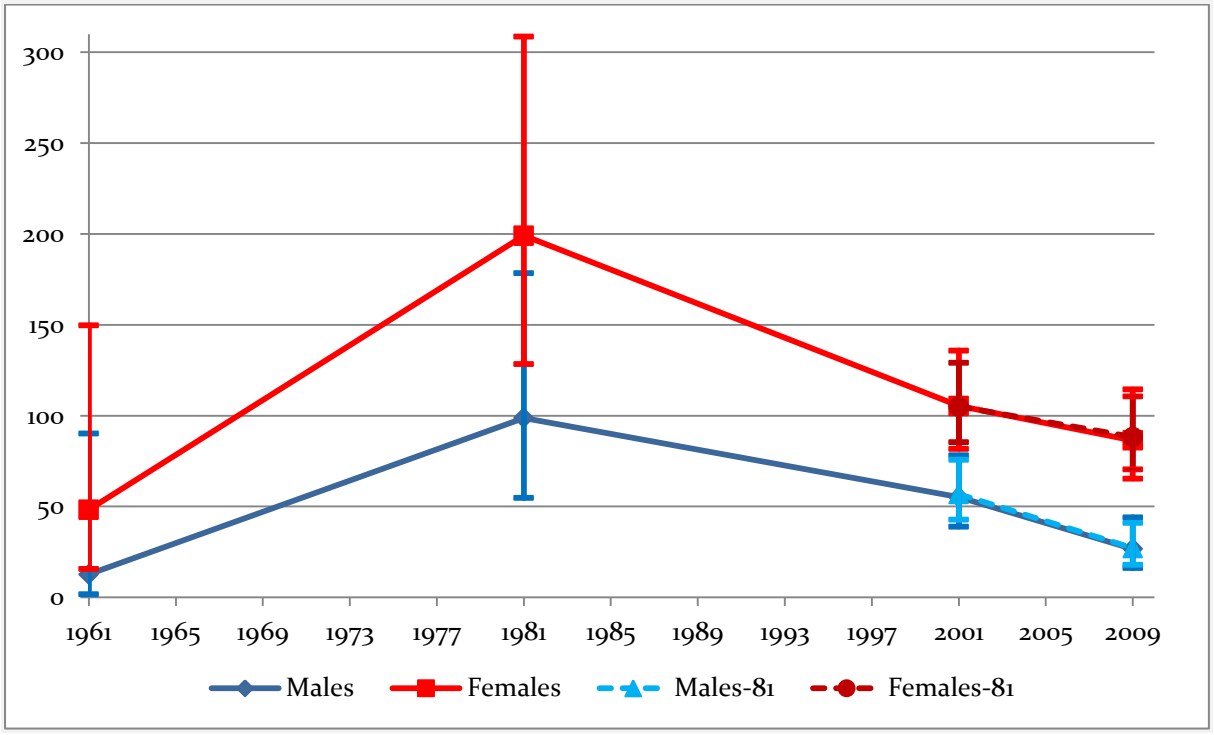
In evaluating the changes in prevalence by sex for the Australian-born and overseas-born prevalence over time, the 1981 values are crude values, since they were estimated as described in detail in Methods note 2.1. It could be argued then, that the spike observed in prevalence by sex and birthplace is an artifact of this failure to age-standardise the 1981 prevalence and that the 1961, 2001 and 2009 prevalence by sex and birthplace, which are age-standardised to the 1961 Greater Hobart population, are incomparable.

To address this concern, we also modelled the prevalence by sex and birthplace with the 2001 and 2009 prevalence age-standardised to the 1981 Australian population. As shown in Methods note Figures A and B, there is very little difference in the age-standardised prevalence in 2001 and 2009 whether they are age-standardised to the 1961 or 1981 Greater Hobart populations. This is doubly informative, as it suggests that the changes between 1961 and 1981 are not an artifact either, indicating that were we able to age-standardise the 1961 prevalence to the 1981 Greater Hobart population, or vice versa, there would be minimal change in their values.

Methods note 2.2 Figure A. Australian-born prevalence by sex and birthplace, with 2001 and 2009 prevalence age-standardised to 1961 Greater Hobart population (solid line) and 1981 Greater Hobart population (dashed line).



Methods note 2.2 Figure B. Overseas-born prevalence by sex and birthplace, with 2001 and 2009 prevalence age-standardised to 1961 Greater Hobart population (solid line) and 1981 Greater Hobart population (dashed line).



Incidence and mortality were age-standardised to the 1954 Hobart population because this population was used to calculate summary measures in the McCall study, which did not provide age-specific rates. The mortality data reported for 1981 are not age-standardised and are not strictly comparable - to assess possible confounding bias, we performed a sensitivity analysis between 1971-81 and 2001-09, standardising the latter to the 1976 Hobart population, the population used to calculate mortality during 1971-81.

Incidence by birthplace was age-standardised to the 1981 Australian population. This population was used to standardise incidence by birthplace in the Hammond study. The McCall study did not report incidence by birthplace.

Sex-specific prevalence and incidence by birthplace for 1971-81 are not age-standardised as these values were not provided as age-specific rates. Sex-specific prevalence by birthplace for 2001 and 2009, and sex-specific incidence by birthplace for 2001-09 were age-standardised to the 1981 Hobart population to allow comparability with the crude sex-specific values from the Hammond study.

2.3.5 Population data

All population data were obtained from the relevant quincennial Census or annual population estimates published by Australian Bureau of Statistics (ABS).(20) All immigration data were obtained from the ABS(20) or the Australian Bureau of Immigration and Multicultural affairs.(21)

2.3.6 Statistical analysis

Poisson regression(19) was used to assess the significance of changes over time in all epidemiological measures - binary (0/1) terms were included as covariates for each 10-year age-group and for sex, while study year was included as a continuous covariate, with population entered as an offset. In assessing the change over time, the coefficient of the year term was used; in assessing the change in the sex ratio, the coefficient of a (sex *x* year) product term was assessed.

To assess the significance of differences in characteristics of the 2001 and 2009 case samples, two-sample t-test and chi-squared test were used.

All statistical analysis was done using STATA/SE for Windows software (Version 10.1; StataCorp LP College Station, TX USA).

2.4 Results

2.4.1 2001 and 2009 studies

In 2001, 326 people were identified as potential MS cases. Of these, 63 were excluded for not having a clinically-definite diagnosis on prevalence day and 47 were not living in the study region, leaving 229 eligible cases. The majority (80.8%) of these were recruited from other MS studies conducted by the Menzies Research Institute, with the remainder recruited by neurologist referral (19.2%).

In 2009, 371 people were identified as potential MS cases. Of these, 43 were excluded for not having a clinically-definite diagnosis on prevalence day and 41 were not living in the study region, leaving 265 eligible cases. The majority of these were prevalent cases in the 2001 study (63.4%), with the remainder recruited by referral from neurologist (10.2%), the local MS Society (23.0%) or from other studies (3.4%).

Table 2.1 shows the characteristics of the 2001 and 2009 cases. Only the mean age changed significantly between 2001 and 2009 ($p=0.01$).

Table 2.1. Characteristics of 2001 and 2009 case samples.

	2001	2009
	n/N (%)	
Sex		
Female	155/229 (67.7)	191/265 (72.9)
Male	74/229 (32.3)	74/265 (27.9)
MS Course		
RRMS[a]	155/229 (67.7)	164/265 (61.9)
SPMS[b]	60/229 (26.2)	74/265 (27.9)
PPMS[c]	14/229 (6.1)	27/265 (10.2)
Australian-born		
Yes	196/229 (85.6)	231/265 (87.2)
No	32/229 (14.0)	33/265 (12.5)
Unknown	1/229 (0.4)	1/265 (0.4)
	Mean (SD; range)	
Age on prevalence day	48.1 (11.8; 20-75)	50.9 (12.7; 18-83)[†]
Age at MS diagnosis	38.6 (10.8; 12-73)	38.3 (11.2; 12-69)
Age at symptom onset[§]	33.2 (9.9; 15-66)	34.0 (10.3; 14-67)
	Prevalence (95% CI)	
Crude prevalence	116.1 (102.0, 132.1)	125.2 (111.0, 141.3)
Age-standardised prevalence[d]	95.6 (79.2, 115.4)	99.6 (82.9, 119.7)
	2001-09	
	Incidence and mortality (95% CI)	
Crude incidence, 2001-09	3.7 (2.8, 4.8)	
Age-standardised incidence[e], 2001-09	3.7 (2.5, 5.4)	
Crude mortality, 2001-09	1.6 (1.1, 2.3)	
Age-standardised mortality[e], 2001-09	1.0 (0.5, 2.2)	

[a] Relapsing-remitting multiple sclerosis; [b] Secondary-progressive multiple sclerosis; [c] Primary-progressive multiple sclerosis; [d] Age-standardised to 1961 Hobart population; [e] Age-standardised to 1954 Hobart population; [†]Significant ($p<0.05$) difference between 2001-09, by t-test or chi-square test, as appropriate; [§]Note: Age at symptom-onset not measured for all persons (28/210 for 2001 cohort; 7/265 for 2009 cohort).

2.4.2 Prevalence: 1961 to 2009

Table 2.2 shows the age and sex-specific prevalence for each of the four studies.

Table 2.2. Age-specific prevalence by sex for Greater Hobart, 1961-2009.

Prevalence per 100,000 with 95% CI. Figure in parentheses is prevalence age-sex standardised to the 1961 Greater Hobart population

Age group (years)	1961 [1]						1981					
	Males		Females		Total		Males		Females		Total	
	Cases	Prevalence /100,000	Cases	Prevalence /100,000	Cases	Prevalence /100,000	Cases	Prevalence /100,000	Cases	Prevalence /100,000	Cases	Prevalence /100,000
0-9	0	0	0	0	0	0	0	0	0	0	0	0
10-19	0	0	2	12.6	2	5.9	0	0	1	6.6	1	3.3
20-29							7	48.7	6	40.7	13	44.7
30-39	3	36.2	7	88.5	10	61.7	13	109.3	19	158	32	133.8
40-49	6	82	9	123.5	15	102.7	9	105.8	17	199.1	26	152.5
50-59	2	37.2	4	75.1	6	56.1	10	117.7	18	211.8	28	164.7
60+	0	0	4	50	4	29.3	4	39.5	21	154.5	25	105.4
Total	11	19.2 (19.2)	26	45.9 (45.9)	37	32.5 (32.5)	43	52.0 (50.7)	82	95.8 (95.8)	125	74.2 (73.1)
95% CI		10.6, 34.6		31.3, 67.5		23.5, 44.8		38.6, 70.1		77.1, 118.9		62.3, 88.5

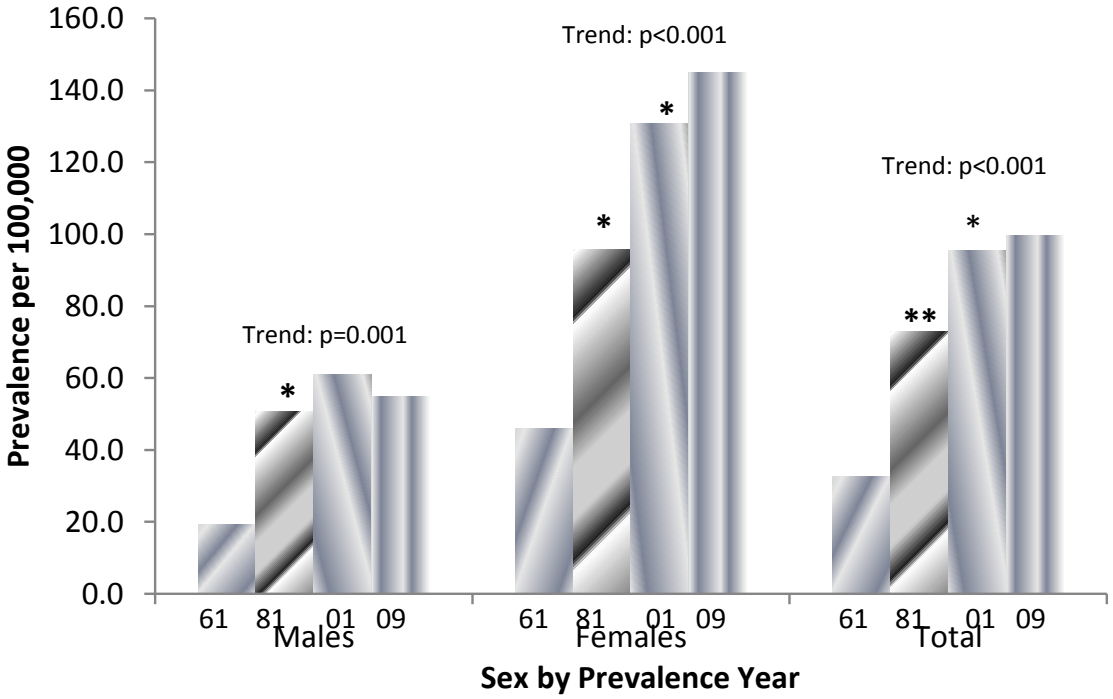
Age group (years)	2001						2009					
	Males		Females		Total		Males		Females		Total	
	Cases	Prevalence /100,000	Cases	Prevalence /100,000	Cases	Prevalence /100,000	Cases	Prevalence /100,000	Cases	Prevalence /100,000	Cases	Prevalence /100,000
0-9	0	0	0	0	0	0	0	0	0	0	0	0
10-19	0	0	0	0	0	0	1	7	1	7.2	2	7.1
20-29	2	15.7	10	76.9	12	46.6	3	20.9	6	41.8	9	31.4
30-39	11	83.3	29	206	40	146.6	7	52.7	31	221.5	38	139.3
40-49	19	131.2	49	321.4	68	228.7	20	140.4	54	352.9	74	250.5
50-59	24	202.1	43	356.6	67	280	19	138.3	58	397.3	77	271.7
60+	18	113.1	24	120.7	42	117.3	24	124.2	41	174.6	65	151.9
Total	74	76.9 (61.0)	155	153.4 (130.7)	229	116.1 (95.6)	74	71.9 (54.8)	191	175.8 (145.0)	265	125.2 (99.6)
95% CI		61.2, 96.6		131.0, 179.5		102.0, 132.1		57.2, 90.3		152.5, 202.6		111.0, 141.3

[1] For the 1961 study, the age-specific counts and prevalence rate were only provided for the 10-29 age group, rather than 10-19 and 20-29 as in the subsequent studies.

Figure 2.3 depicts the age-standardised prevalence values by sex over the four studies. There was a significant increase in the total ($p<0.001$), male ($p=0.001$) and female ($p<0.001$) prevalence between 1961 and 2009. The majority of this change occurred between 1961 and 2001, while the change between 2001 and 2009 was not significant ($p=0.87$).

Figure 2.3. Prevalence by sex for Greater Hobart: 1961-2009, age-standardised to the 1961 Greater Hobart population.

Significance of change from previous study assessed by Poisson regression. [* p<0.05; ** p<0.001]



2.4.3 Prevalence by birthplace: 1961 to 2009

Table 2.3 shows the prevalence by sex and birthplace for each of the four studies; Figure 2.4 shows the total prevalence over time by birthplace. The total prevalence among Australian-born has been consistently rising since 1961 ($p<0.001$). Overseas-born prevalence also increased between 1961 and 2009 ($p=0.05$), however this occurred via a significant increase between 1961 and 1981 ($p<0.001$), before falling between 1981 and 2001 ($p=0.31$) and between 2001 and 2009 ($p=0.16$). Standardising to the 1981 population, rather than the 1961 population, did not alter these findings.

Table 2.3. Age-specific prevalence by sex and by birthplace for Greater Hobart, 1961-2009, all prevalence values standardised to 1961 Greater Hobart population.

Note: Prevalence values for 1981 are not age-standardised to the 1961 population like the other years, but are presented as crude as age-specific prevalence data were not available for age-standardisation.

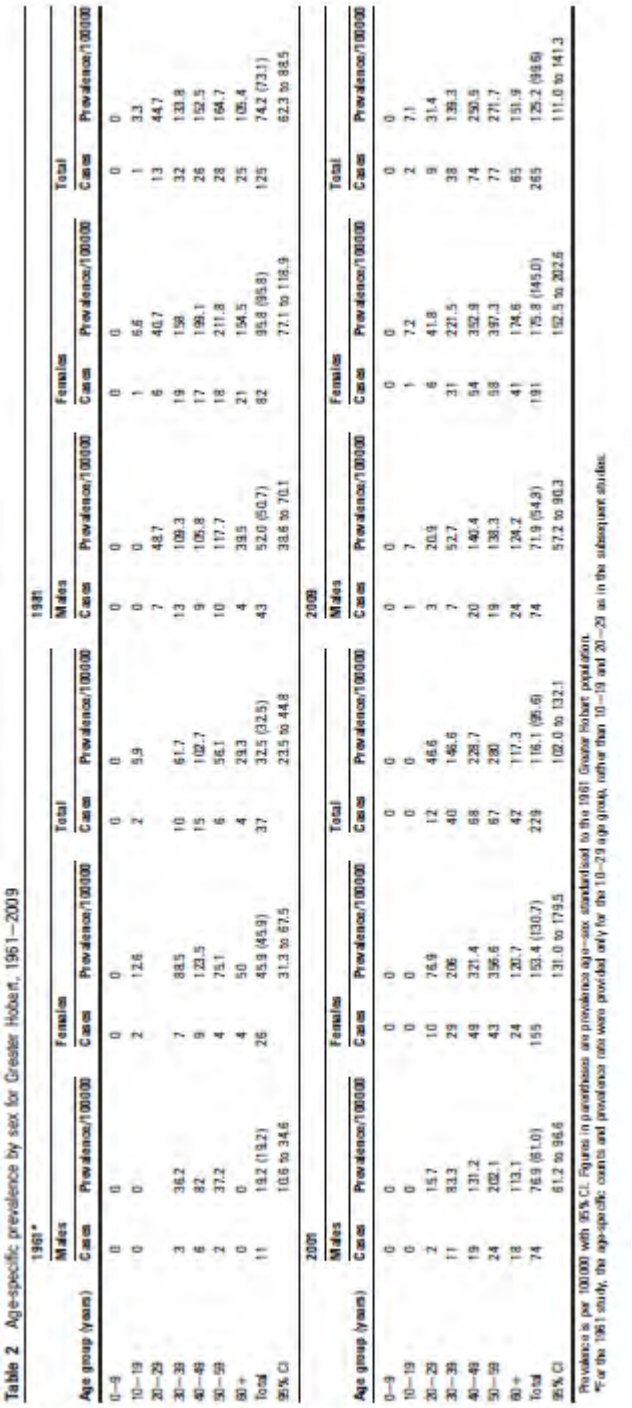


Table 2 Age-specific prevalence by sex for Greater Hobart, 1961–2009

	1961*						1981					
	Males		Females		Total		Males		Females		Total	
Age group (years)	Cases	Prevalence/100000	Cases	Prevalence/100000	Cases	Prevalence/100000	Cases	Prevalence/100000	Cases	Prevalence/100000	Cases	Prevalence/100000
0–9	0	0	0	0	0	0	0	0	0	0	0	0
10–19	0	0	2	12.6	2	5.9	0	0	1	6.6	1	3.3
20–29							7	48.7	6	40.7	13	44.7
30–39	3	36.2	7	88.5	10	61.7	13	109.3	19	158	32	133.8
40–49	6	82	9	123.5	15	102.7	9	105.8	17	199.1	26	152.5
50–59	2	37.2	4	75.1	6	56.1	10	117.7	18	211.8	28	164.7
60+	0	0	4	50	4	29.3	4	39.5	21	154.5	25	106.4
Total	11	19.2 (19.2)	26	45.9 (45.9)	37	32.5 (32.5)	43	52.0 (60.7)	82	95.8 (95.8)	125	74.2 (73.1)
95% CI		10.6 to 34.6		31.3 to 67.5		23.5 to 44.8		38.6 to 70.1		77.1 to 118.9		62.3 to 88.5

	2009						2009					
	Males		Females		Total		Males		Females		Total	
Age group (years)	Cases	Prevalence/100000	Cases	Prevalence/100000	Cases	Prevalence/100000	Cases	Prevalence/100000	Cases	Prevalence/100000	Cases	Prevalence/100000
0–9	0	0	0	0	0	0	0	0	0	0	0	0
10–19	0	0	0	0	0	0	1	7	1	7.2	2	7.1
20–29	2	15.7	10	76.9	12	46.6	3	20.9	6	41.8	9	31.4
30–39	11	83.3	29	206	40	146.6	7	52.7	31	221.5	38	139.3
40–49	19	131.2	49	321.4	68	228.7	20	140.4	54	352.9	74	250.5
50–59	24	202.1	43	356.6	67	280	19	138.3	58	387.3	77	271.7
60+	18	113.1	24	120.7	42	117.3	24	124.2	41	174.6	65	151.9
Total	74	76.9 (81.0)	155	153.4 (130.7)	229	116.1 (95.6)	74	71.9 (64.9)	191	175.8 (145.0)	265	125.2 (98.6)
95% CI		61.2 to 96.6		131.0 to 179.5		102.0 to 132.1		57.2 to 90.3		152.5 to 202.6		111.0 to 141.3

Prevalence is per 100000 with 95% CI. Figures in parentheses are prevalence age–sex standardised to the 1961 Greater Hobart population.
*For the 1961 study, the age-specific counts and prevalence rate were provided only for the 10–29 age group, rather than 10–19 and 20–29 as in the subsequent studies.

Figure 2.4. Prevalence by birthplace for Greater Hobart: 1961-2009, age-standardised to the 1961 Greater Hobart population†.

Error bars show 95% confidence intervals. †Note: Prevalence values for 1981 are not age-standardised to the 1961 population like the other years, but rather to the 1981 Australian population as this was the value standardised to in the Hammond study.

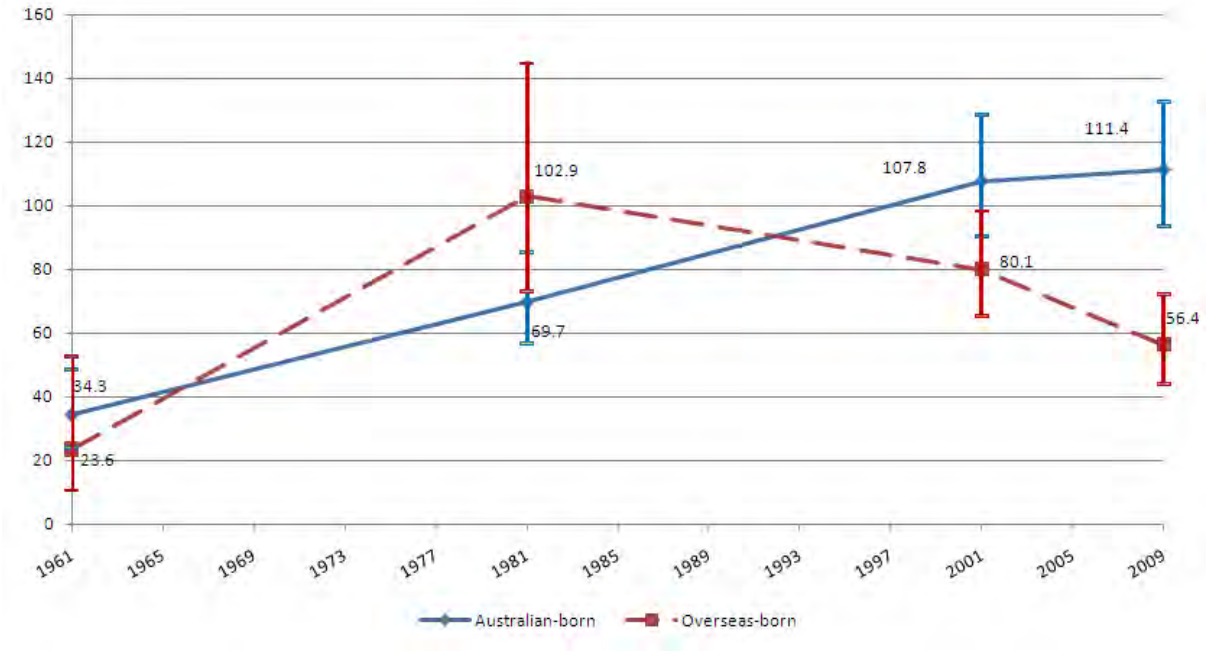
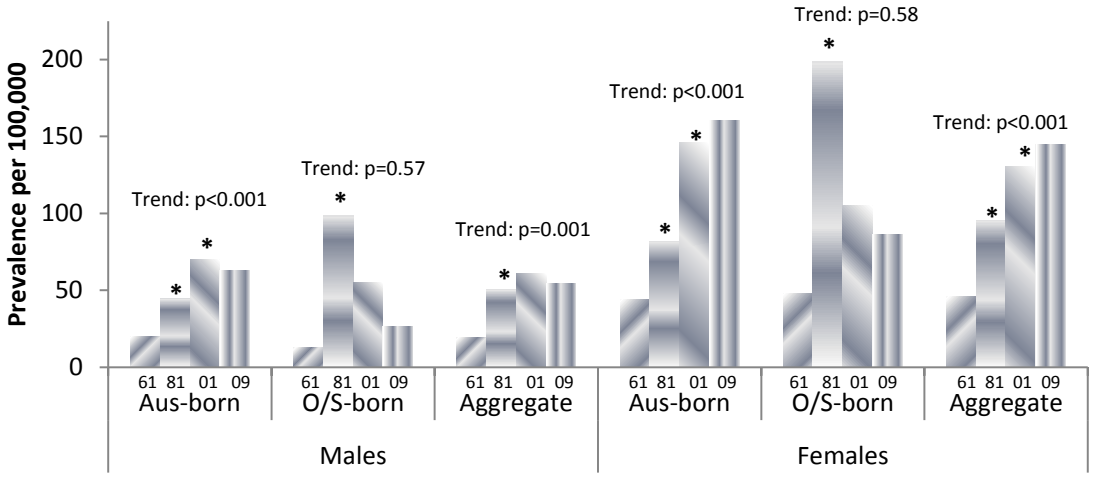


Figure 2.5 shows the change in prevalence for males and females by birthplace and for the aggregate. The patterns of change for males and females are similar. The changes in the aggregate reflect those of the Australian-born, due to the much larger numbers of cases contributed by this source. Of note is the difference in trend by sex among the Australian-born between 2001 and 2009, with males deceasing while females continue to increase, though neither of these changes reach statistical significance.

Figure 2.5. Aggregate prevalence and prevalence by birthplace, by sex for Greater Hobart: 1961-2009, age-standardised to the 1961 Greater Hobart population†.

Significance of change from previous study assessed by Poisson regression. [* p<0.05] †1981 values not age-standardised.



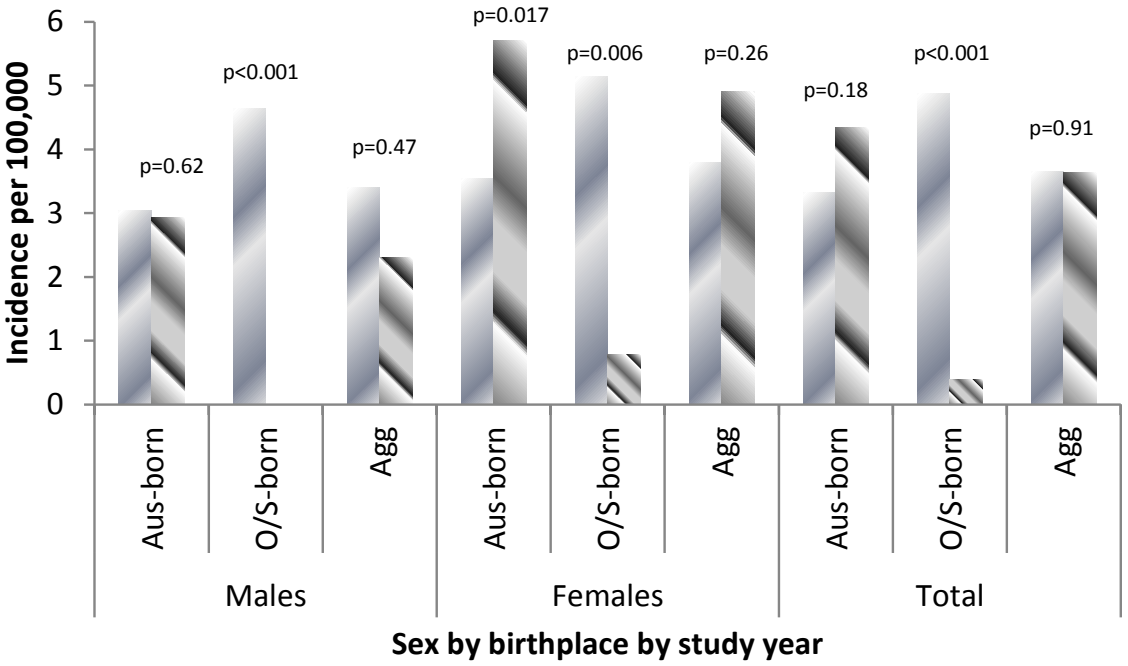
2.4.4 Incidence: 1951-61 to 2001-09

The total aggregate incidence increased significantly between 1951-61 and 2001-09 (p=0.04), from 2.2 in 1951-61, to 3.6 in 1971-81 and 3.7 in 2001-09. However this change was confined to the first two periods (p=0.04), with no significant change thereafter (p=0.91).

Figure 2.6 shows the change in incidence by sex and birthplace between 1971-81 and 2001-09. While total and aggregate incidence remained unchanged between 1971-81 and 2001-09, the incidence for overseas-born groups decreased significantly (p<0.001 for males and total; p=0.006 for females). Among Australian-born, only female incidence increased significantly (p=0.017), while males and the total were statistically unchanged.

Figure 2.6. Incidence rates for Greater Hobart by birthplace, by sex for 1971-81 and 2001-09 study periods.

2001-09 incidence rates age-standardised to the 1981 Greater Hobart population†. Significance assessed by Poisson regression. †1971-81 incidence not age-standardised.

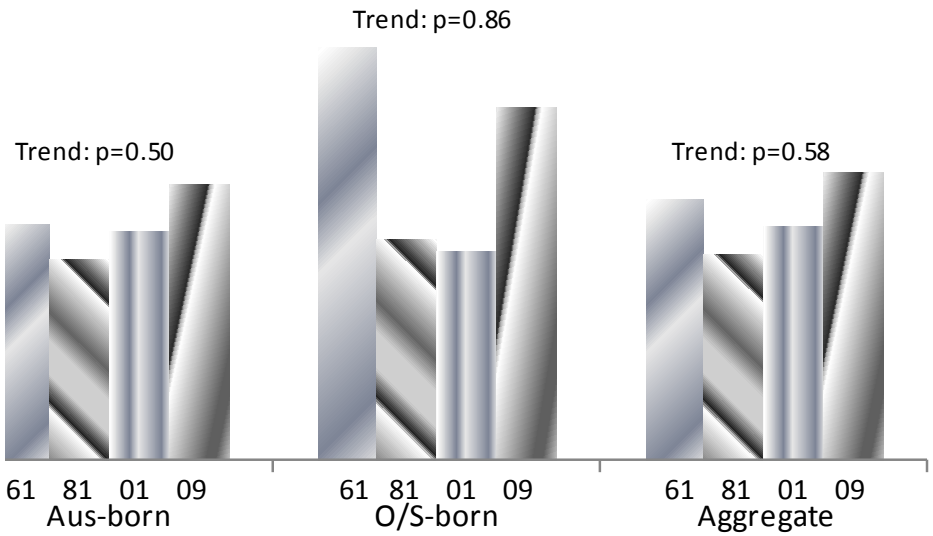


2.4.5 Prevalence and incidence sex ratios: 1951 to 2009

As shown in Figure 2.7, there was no change in the aggregate prevalence sex ratio (PSR) between 1961 and 2009 (p=0.48), nor in the Australian-born PSR (p=0.63) or overseas-born PSR (p=0.34).

Figure 2.7. Prevalence sex ratio (female/male) for prevalence by birthplace and for the aggregate.

Significance assessed by Poisson regression.



The incidence sex ratio (ISR) increased between the 1971-81 and 2001-09 study periods, from 1.1 (95% CI: 0.8, 1.6) to 2.1 (95% CI: 1.4, 3.0), however this was not significant (p=0.18). The ISR among the Australian-born increased near-significantly (p=0.06) from 1.2 (95% CI: 0.9, 1.6) to 1.9 (95% CI: 1.5, 2.5). Among the overseas-born, the ISR was 1.1 (95% CI: 0.6, 2.1) in 1971-81; the 2001-09 ISR was undeterminable as the male overseas-born incidence in 2001-09 was zero.

2.4.6 Mortality and longevity

The mortality rate decreased from 2.4/100,000 in 1951-59 to 1.1/100,000 in 1971-81 (p=0.02), and remained statistically unchanged thereafter (1.0/100,000 in 2001-09, p=0.81). The mean age of the prevalent cohort increased significantly between 1961 to 2009, from 41.0 to 46.4 years (p=0.02). This increase was stronger among males, increasing from 40.1 to 47.8 years (p=0.01); for females the mean age increased from 41.4 to 45.9 years (p=0.26).

2.5 Discussion

We have undertaken a two-stage study of MS frequency in Hobart between 2001 and 2009 and, in combination with previously published studies, have conducted a time-trend analysis of MS epidemiology in Greater Hobart over the 58-year period from 1951 to 2009. This is the longest such study of MS frequency in the Southern hemisphere and comparable in length with the longest study durations in the Northern hemisphere.(22, 23) We found high rates of MS, with crude prevalence of 116.1/100,000 in 2001 and 125.2/100,000 in 2009, a marked increase from the 1961 prevalence of 32.5/100,000. This persists after age-standardisation, now a three-fold increase to a 2009 prevalence 99.6/100,000, increasing by 14/100,000 every 10 years. The significant effect of age-standardisation reflects the aging of the Hobart population over this interval and accounts for a portion of the increase in prevalence.

Over 2001-09, we observed a crude incidence of 3.7/100,000, nearly double the 1951-61 incidence of 2.2/100,000; this increase persists on age-standardisation. Importantly, we observed significant differences by birthplace in the trends in both prevalence and incidence. Australian-born prevalence and incidence showed a steady increase over time, while overseas-born prevalence and incidence increased markedly up to 1981, whereupon prevalence and incidence fell precipitously. The 2001-09 age-standardised mortality rate was 1.0/100,000, less than half that of the 1951-61 period (2.4/100,000). This reduced mortality, along with a significant increase in the mean age of the prevalent cohort, from 41.0 to 46.4 years, manifest in increased case longevity.

2.5.1 Epidemiology by place

Hobart (42.9°S) had the highest reported prevalence and incidence of MS in Australia at all time points. In the current study, the 2001 Hobart prevalence (116.1/100,000) was nearly double that of Newcastle (32.9°S) in 1996 (59.1/100,000).(8) The incidence for the 2001-09 period (3.7/100,000) was 1.5-times higher than the 1986-96 Newcastle incidence (2.4/100,000)(8); this

persisted on age-standardisation. Globally, Hobart's 2009 age-standardised prevalence (99.6/100,000) is comparable to those found in contemporaneous studies at similar latitudes: the West Coast/Canterbury(9) (42.3°S) region of New Zealand in 2006 (86.6/100,000), the Otago/Southland(9) (46.2°S) region of New Zealand in 2006 (109.4/100,000) and Ferrara province(24) (44.8°N) in Italy in 2004 (95.0/100,000).

2.5.2 Epidemiology over time

We observed a three-fold increase in the age-standardised prevalence from 1961 to 2009. Such increases in prevalence over time are common in serial prevalence studies.(4, 5, 8, 9, 25) Poskanzer(26) suggests that an increase in prevalence in a serially-measured population may reflect increased incidence, differential emigration between cases and non-cases, changes in population-structure, increased duration of disease and/or changes in case ascertainment. In our study area, between 1951-61 and 2001-09 the age-standardised incidence increased from 2.2/100,000 to 3.7/100,000, contributing to increased prevalence (Figure 3.6). We have no information regarding differential migration between cases and non-cases, however we also have no reason to assume such a difference. Figure 3.2 demonstrates there has been a considerable change in the Hobart population structure, but this has been addressed by age-standardisation. Along with an aging of the population however, there has been an increase in the mean age of the prevalent MS cohort which, acting in concert with the decreased mortality, manifests in a significantly increased duration of disease in Hobart, accounting for a further component of the prevalence increase.

Specialist neurological care has been provided for MS cases in Hobart throughout the study period and it is unlikely that changes in prevalence and incidence can be traced to differences in this part of case ascertainment. However, changes in the diagnostic criteria used, from the Allison and Millar criteria used by McCall and colleagues and the Rose criteria used by Hammond and colleagues, to the 2001 and 2005 McDonald criteria used in our prevalence studies, do impact prevalence estimates. In one aspect,

the increase in prevalence may be partially due to the increased sensitivity of diagnostic criteria over time, allowing for the detection of less "typical" cases, particularly progressive courses which were not explicitly allowed for under the Allison & Millar criteria. Use by modern criteria of paraclinical evidence such as MRI allows for the inclusion of such cases. At the same time however, the lower specificity of the earlier criteria, reliant solely on clinical evidence and history, allowed the inclusion of similar, but non-MS neurological conditions as "possible" MS, a group which was included in the McCall and Hammond calculations of prevalence and incidence. Studies comparing prevalence using these criteria with the 1984 Poser criteria(27) showed a higher prevalence with the Allison and Millar criteria (8.7% to 18.4%)(28, 29) and the Rose criteria (1.6% to 11.9%)(7, 30). Of note, studies comparing the Poser(27) and 2001 McDonald(15) criteria (the criteria used in our 2001 study), found little (0.7%) or no difference in their diagnostic allocation(31, 32); a study(33) comparing the 2001 & 2005 McDonald criteria (the criteria used in our 2001 and 2009 studies) found no difference in their specificities. Therefore the McCall and Hammond studies likely overestimated prevalence, resulting in an estimate of the increase in prevalence and incidence to 2009 which is actually lower than that actually occurring.

2.5.3 The effects of migration

There were significant differences in the changes in prevalence and incidence over time by birthplace. Among Australian-born, there was a steady increase in prevalence and incidence over all study points; among the overseas-born, there were significant increases in the prevalence and incidence up to 1981, whereupon there were significant declines, prevalence falling precipitously after 1981, and incidence being reduced to near zero by 2001-09. These findings may be explained by the Australian assisted-migration scheme of 1945-81. Australia was founded and largely populated by immigrants from the UK.(34) Following the Second World War, Australia sought to drastically increase the population and the Australian government began providing assisted-migration, from 1945 until 1981.(34) Over this period, 3.8 million migrants came to Australia, the largest fraction (38.8%) coming from the UK(20,

34), and the majority (52.8%) of them male.(20, 34) After the termination of assisted-migration in 1981, the demographics of immigrants shifted, changing from mostly coming from high-prevalence(13, 14) and higher latitude (average: 42.92°) nations in Europe and North America (82.2%) to mostly from low-prevalence(13, 14) and low latitude (average: 28.42°) nations (57.6%), particularly in Asia/Oceania (32.8%)(34).

The effects of assisted-migration are now fading, as evidenced by the decreasing prevalence among the overseas-born in 2001 and 2009 (Figures 4,5) and the sharply decreased overseas-born incidence in 2001-9 (Figure 3.6). However, this mass influx of genetically-susceptible immigrants up to 1981, and its abrupt cessation thereafter, had significant effects on MS epidemiology in Hobart over 1951-2009. Therefore, MS epidemiology in Hobart over 1951-2009 is actually a tale of two populations. Only upon extricating these two populations from one another can sense be made of the whole and assumptions about future trends made – such predictions of future trend are important in planning hospital and health services.

Discussion note 2.1. Change in Australian immigration patterns over time, pre/post-assisted migration

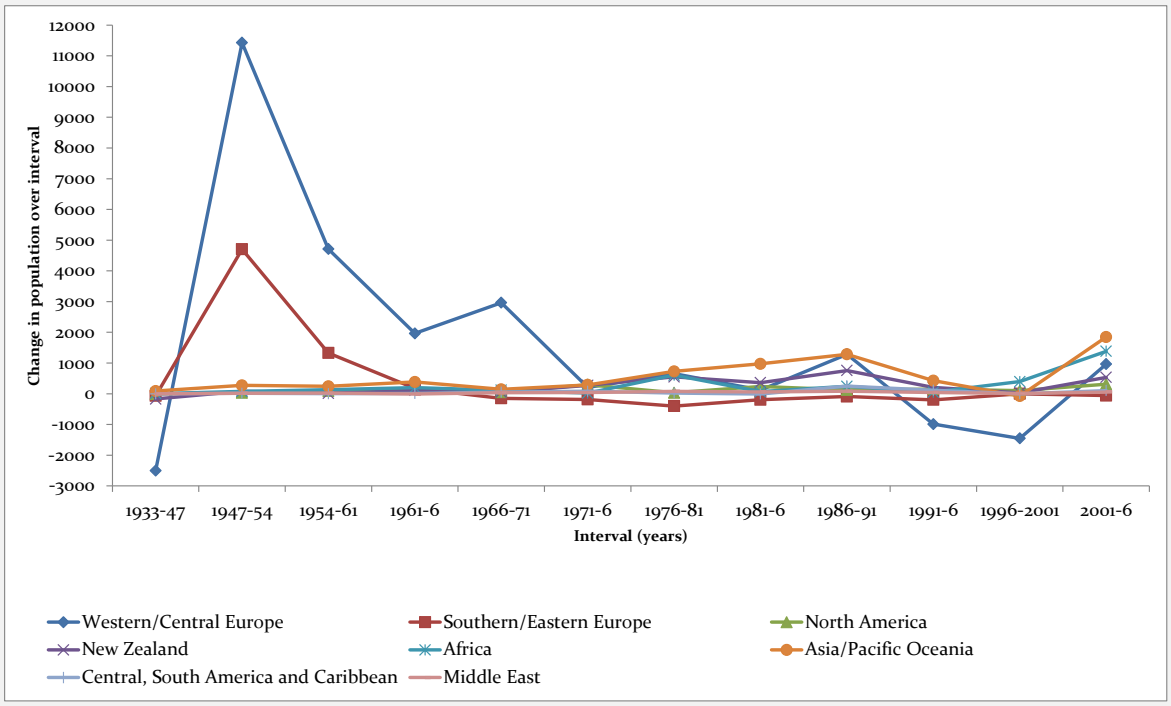
We assert in the Discussion that some of the change in the prevalence sex ratio over time may be due to changes in the distribution of immigrants to Australia, before and during the Assisted Migration scheme of 1945-1981 and thereafter. We note that the majority of immigrants were from the UK, but do not provide evidence for this. We do that here.

Exact country-of-origin information for migrants at the state level were not available for the time period examined. However we do have country-of-birth information at the state level for each of the quinquennial censuses, and by subtracting the values between censuses, we get an estimate of the immigration from given countries during the intervening period. Examining this in Discussion note 2.1 Figure A, we immediately see the advent of the assisted migration scheme, with the noteable spike in

persons of Western and Central European birth between 1933-47 and 1947-54, alongside a lesser but still significant spike in persons of Southern and Eastern European birth; of additional note is that this spike of European immigration into Tasmania effectively ends in the early 1970s.

Discussion note 2.1 Figure A. Change in immigration to Tasmania, all persons, by *global* region of origin.

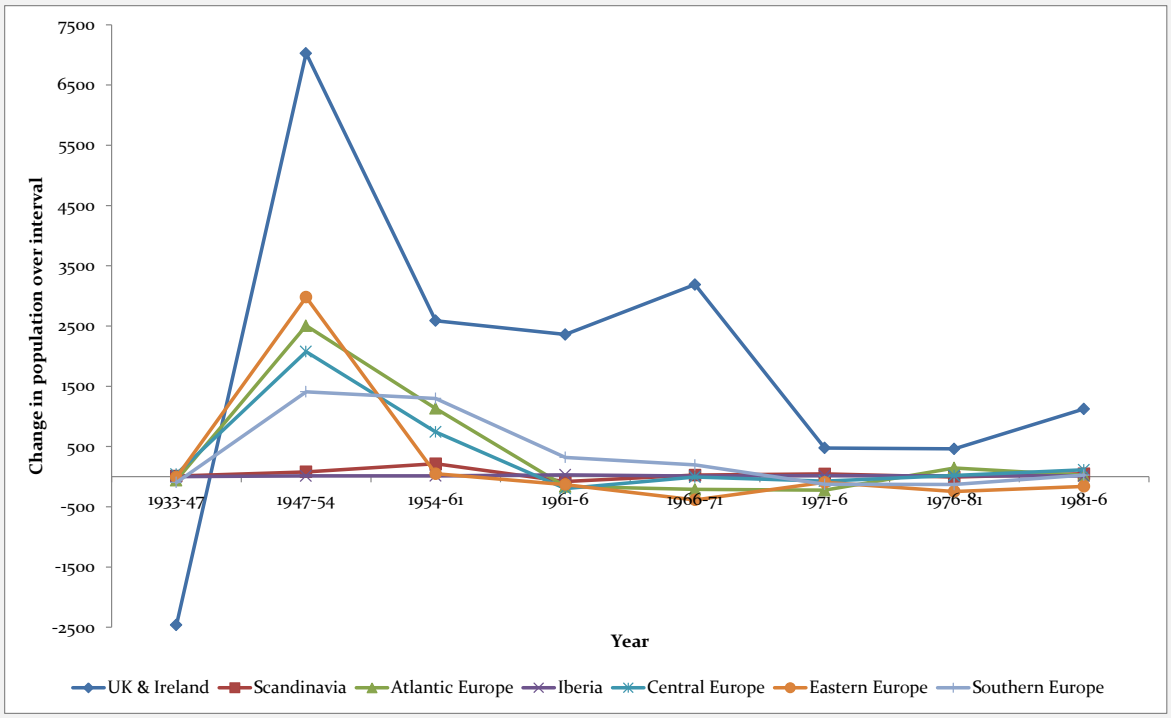
(Data obtained from Australian Bureau of Statistics)



Examining the changes in the distribution of immigrants to Tasmania from regions within Europe, it is apparent that the overwhelming majority of immigrants are from the UK (Discussion note 2.1 Figure B). While in the first 5-year interval of the assisted migration program there is some additional influx from countries outside the UK, the immigrants from these nations fall off immediately, effectively reaching a net influx of 0 by the mid-1960s. For the UK, however, while the number of immigrants also falls off after the initial spike in 1947-54, a high level of migrants persist until the mid-1960s, whereupon they are much reduced. Similar trends are observed by sex (not shown).

Discussion note 2.1 Figure B. Change in immigration to Tasmania, all persons, by *European* region of origin.

(Data obtained from Australian Bureau of Statistics)

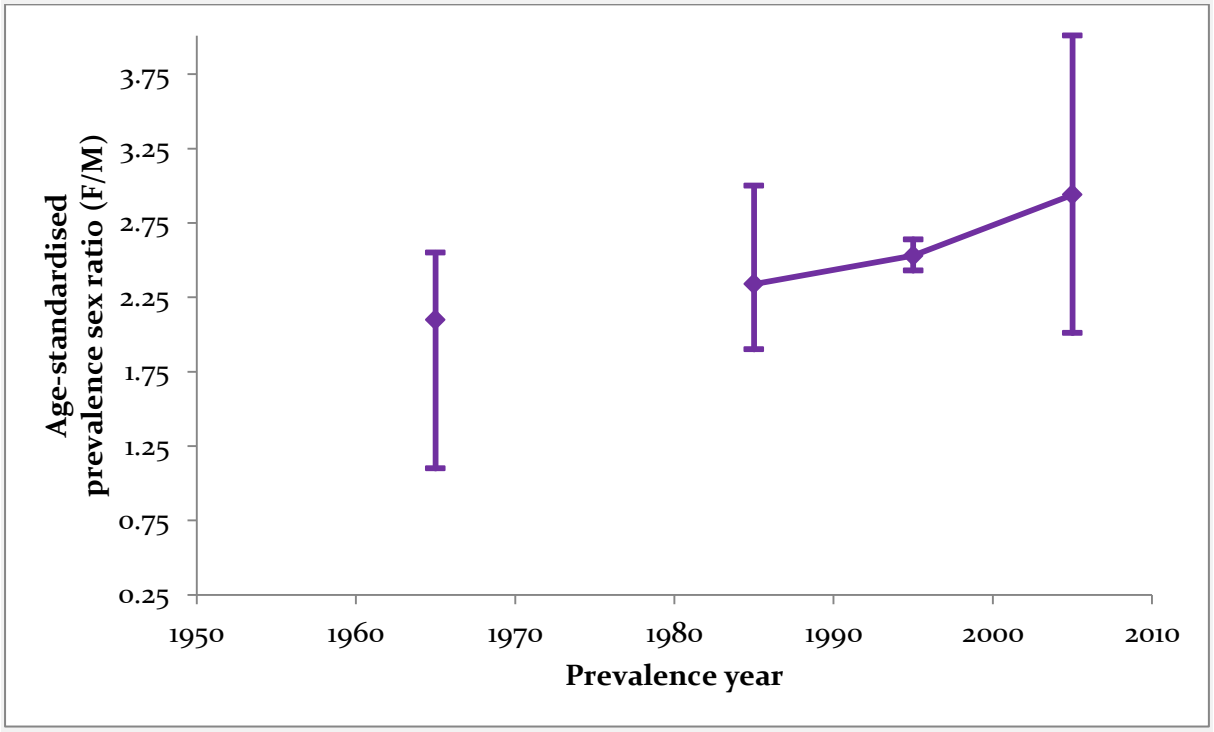


Of note, however, are the prevalence sex ratios in the regions over time, this information gleaned from prevalence studies in these regions over time (See Chapter 3). As in Discussion note Figure 3A and 3B, the age-standardised prevalence sex ratios from prevalence studies undertaken in the Australasia region (Australia/New Zealand) in the 1940s-1960s are higher than those of Western Europe (UK, Ireland, France, Belgium, Netherlands, Denmark, Sweden, Norway, Finland, Italy and Malta).

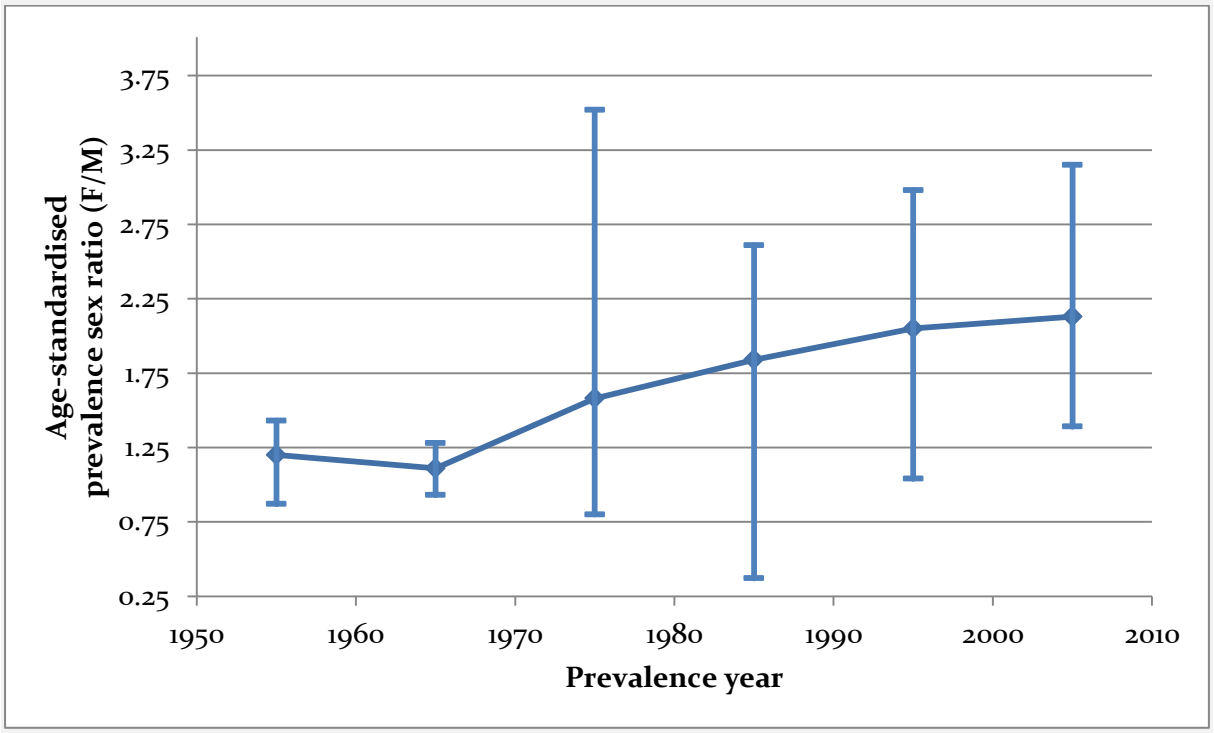
Discussion note 2.1 Figure C. Prevalence sex ratio (female/male), prevalence age-standardised to 2009 European population.

(Data obtained from Australian Bureau of Statistics)

a) Australasia



b) Western Europe.



While there is a paucity of studies reporting age and sex-specific prevalence from this period, what data we have finds the mean prevalence sex ratio for Australasia at this point to be around 2.0, while in Western Europe it is around 1.25. This might suggest that this influx of persons from Western Europe during the assisted migration period might "bring with them" their lower sex ratio. This, in combination with the influx of males to the small population of Hobart, might have acted to drive down the prevalence sex ratio measured in 1980. After the cessation of the assisted migration scheme, and the shift in migrants to come from low-risk areas in Asia, the local Australian-born population and its higher prevalence sex ratio came to the fore as the overseas-born persons from Europe and their immediate progeny expire or emigrated.

2.5.4 Sex ratios over time

We found no significant change in the age-standardised PSR over 1961-2009 - this is in line with other studies.(4, 8, 9, 25, 35-38) Only in one location has a significant change in the age-standardised PSR been found, in Ferrara province, Italy between 1978(39) and 2004(24), increasing from 1.1 to 2.4 ($p<0.05$).

Among the Australian-born, we observed a near-significant ($p=0.06$) increase in the ISR between 1971-81 and 2001-9, increasing from 1.2 to 1.9. In the aggregate, we found a trend to increased ISR, nearly doubling from 1.1 to 2.1. While this trend did not reach significance, this likely reflects the small number of incident cases in both periods, with insufficient power resulting in a lack of statistical significance. Certainly elsewhere, significant changes in the ISR have been observed(40): in Canada(41), the ISR increased significantly over 50 years (1.014 per year over 1931-1980). In Oslo(22), the ISR increased significantly, from 1.48 to 2.30 over 1910-80. While our data do show a smaller increase in the ISR than that observed by others(8, 22, 41), the trend to increased ISR is in keeping with the literature(40). It may be that this smaller magnitude of the change in ISR is a reflection of a differential effect of latitude on MS risk by sex. Such a result is borne out in our recent

work(42), where the ISR of first demyelinating events varied inversely with latitude within Australia, with a 2.7-times higher sex ratio in Brisbane (27.3°S) than Hobart (42.8°S).

2.5.5 Strengths and limitations

A key strength of this study is the long time-span that was studied. The medical infrastructure and specialist neurological care available throughout the period makes it possible to largely discount the possibility of differential case ascertainment. Also, the relatively high number of cases available for study in a stable and geographically-confined, genetically-susceptible population makes this an ideal location for long-term study of MS epidemiology.

There were some limitations in our study. We were restricted to the data presented in the McCall and Hammond publications, thus requiring diverse age-standardisations as described in the Methods. However when sensitivity analyses were undertaken these assumptions did not significantly alter any outcomes.

2.5.6 Conclusion

Our results show a continuing increase in the prevalence and incidence of MS in Greater Hobart, particularly when the analysis is restricted to Australian-born. In our study area, between 1951-61 and 2001-09 the age-standardised incidence increased from 2.2/100,000 to 3.7/100,000. As well as this increase in incidence, increased longevity and a decreased mortality rate have significantly contributed to the increasing prevalence and have manifested in a significantly older cohort. While our data show a trend to increased ISR, this increase is much smaller and less significant that that observed elsewhere, suggesting a differential relationship between prevalence and latitude by sex.

2.6 Summary

Background: Hobart, Tasmania has been the site of two major studies of multiple sclerosis (MS) frequency, in 1951-61 and 1971-81. Since then, there have been no studies of MS frequency in Hobart.

Methods: Using a prevalent cohort of 226 cases in 2001 and 265 in 2009, we undertook a two-stage survey of MS frequency in Hobart. Combined with the published data from the two preceding studies, we conducted a time-trend analysis of MS epidemiology over 1951-2009.

Results: The age-standardised prevalence in 2001 was 96.5/100,000, and 99.5/100,000 in 2009, a significant increase from the 1961 prevalence of 32.5/100,000 ($p<0.001$). Female prevalence increased over each time point; male prevalence increased between 1961 and 2001 but was unchanged thereafter. Incidence over 2001-09 was 3.7/100,000, significantly increased from the 1951-61 incidence of 2.2/100,000 ($p=0.004$), though the majority of this was between 1951-61 and 1971-81. Mortality fell by half from 2.4/100,000 in 1951-59 to 1.0/100,000 in 2001-09 – this decreased mortality and an older cohort contributes to the increase in prevalence. Neither prevalence ($p=0.48$) nor incidence ($p=0.18$) sex ratios changed significantly between 1951 and 2009.

Conclusions: Between 1951 and 2009, the age-standardised prevalence of MS in Hobart increased three-fold and the incidence nearly doubled. Part of the increase in prevalence was due to an increased longevity, decreased mortality and increased incidence. Differences in patterns by birthplace may be explained by the Australian assisted-migration program of 1945-81. Our data do not demonstrate the strong and significant changes in sex ratio observed elsewhere.

2.7 Postscript

We have demonstrated that the Greater Hobart area continues to have the highest prevalence and incidence of MS in Australia, in keeping with the preceding studies. However, whereas the total prevalence has increased significantly since the last measure in 1981, the total aggregate incidence rate is unchanged. This in combination with a markedly reduced mortality rate and increased longevity might lead to the conclusion that MS in the area has plateaued over the last few decades, with increased prevalence merely reflecting an ageing prevalent population. It is only on evaluating the sex and birthplace-specific trends that the true changes over time are revealed, demonstrating the complex interplay of the novel migration patterns of the mid-20th century acting alongside a changing incidence sex ratio among the Australian-born since 1981. While the increase in prevalence over time is due in part to increased survival, the incidence rate among Australian-born females has increased significantly since 1971-81, while that of males has indeed held static. With the effects of emigration and mortality acting upon the prevalent population with MS, the effects of this shift in incidence will yield an increasingly female cohort. The change in the incidence sex ratio among the Australian-born between 1971-81 and 2001-9 did not quite reach statistical significance; however the strong evidence of a trend, alongside similar findings in Europe and North America of an increasing sex ratio over time, suggest that MS in Hobart will increasingly become a disease of females. This, along with the ageing of the population, portend a markedly altered epidemiology than that observed by McCall and colleagues half a century ago, and with significant implications for allocation of health resources and for future medical practitioners.

2.8 References

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Appendix 2A. Publication of "Trends in the epidemiology of multiple sclerosis in Greater Hobart, Tasmania: 1951 – 2009"

Simpson, Jr. SL, Pittas F, van der Mei I, Blizzard L, Ponsonby A-L, Taylor B. "Trends in the epidemiology of multiple sclerosis in Greater Hobart, Tasmania: 1951 to 2009." *Journal of Neurology, Neurosurgery & Psychiatry*. Feb 2011; 82(2): 180-187.

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copyright or proprietary reasons.

Chapter 3. Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis

3.1 Preface

The preceding chapter demonstrated that Hobart has and continues to have the highest prevalence and incidence rates in Australia, nearly twice that of the sister cities of the 3-city studies of Newcastle, NSW and Perth, WA, and nearly 7-times that of northern Queensland. This increased frequency of MS with increasing latitude, while archetypical in Australia, is not unique to it. Indeed, physicians and local epidemiologists have long noted the tendency to find higher frequencies of MS at higher latitudes, both supra-nationally across Europe and North America, as well as within nations. While the latitudinal gradient hypothesis has engendered hypotheses implicating roles for personal UVR exposure and vitamin D in mediating MS onset, various reviews have suggested that rather than reflecting geophysical features that vary with latitude, particularly UVR, instead the gradient is merely an artifact of methodological differences between studies, or the selective migration of genetically-susceptible persons to higher latitude regions. More recently, a meta-analysis suggested that the prevalence gradient did not in fact exist globally.

A definitive evaluation of the latitudinal gradient hypothesis was needed, so as to assess whether a prevalence gradient with increasing latitude existed. This chapter then will discuss my work in evaluating the distribution of global and intraregional prevalence with latitude, its presence and magnitude. Further, I analyse the notable exceptions to the gradient, in northern Scandinavia and Mediterranean Europe, and attempt to reconcile these aberrations with the global gradient. This chapter was published in Journal of Neurology, Neurosurgery & Psychiatry (Appendix 3F). The methods notes (grey boxes) are added for this thesis and were not part of the original publication.

3.2 Introduction

It has long been recognized that there is a distinct latitudinal variation in multiple sclerosis (MS) frequency, higher latitude correlating with increased prevalence, incidence and mortality rates. Understanding the geoepidemiology of MS can be a valuable source of environmental and genetic aetiological clues. MS geoepidemiology has thus become a major research focus, and the latitudinal gradient hypothesis a point of contention(1-6). While gradients have been demonstrated in Australasia(7, 8), Japan(9), Europe(10), and North America(11), other studies(12, 13) have found no association between prevalence and latitude. Also, studies in Mediterranean Europe have found higher-than-expected prevalence for their latitudes, whilst studies in northern Scandinavia(14) have found lower-than-expected prevalence. This has led some(3, 4, 6) to suggest that the gradient is an artifact.

While individual studies have provided evidence, the only way to evaluate the geoepidemiology of MS is to combine findings from a number of studies and there have been few of these. Early work by Kurtzke(1) described bands of high, medium and low frequency, later revised to vary with longitude(2). However, in a 1994 review of MS epidemiology in Europe(15) and a 2001 review globally(3), Rosati argued that the linear gradient hypothesis was an oversimplification, pointing particularly to studies undertaken in Mediterranean Europe after 1980 which found high prevalence in a Kurtzke medium-prevalence zone(1, 2), and instead proposed that much of the variation in frequency was due to different genetic susceptibilities.

The first meta-analysis of MS geoepidemiology was done by Zivadinov and colleagues in 2003(4), combining data from 69 prevalence and 22 incidence estimates between 1980 and 1998. Importantly, in addition to analysing crude values, Zivadinov age-standardised prevalence, reporting a significant gradient in the crude analysis that was attenuated on age-standardisation. The authors reported no association between latitude and incidence was found after age-standardisation however.

In 2008, Alonso and Hérnan undertook a meta-analysis of MS incidence, including 38 age-standardised incidence estimates between 1966 and 2007(5). These authors found that, in contradistinction to the findings by Zivadinov(4), there was a significant association between incidence and latitude, though moderated after 1980.

Recently, Koch-Henriksen and Sørensen(6) published findings from a meta-analysis of 97 crude MS prevalence and 122 incidence estimates, reporting "modest" associations between prevalence and latitude in Western Europe and North America. The authors found no association between incidence and latitude within Western Europe or North America. Surprisingly, in Australasia, an archetype of the latitudinal gradient(7, 8), the authors reported that there was no association between latitude and prevalence, nor incidence after adjusting for study prevalence-year.

The systematic reviews of MS prevalence geoepidemiology(4, 6), particularly that by Koch-Henriksen and Sørensen(6), had some significant methodological shortcomings that may have influenced their results. Further, in light of our own findings regarding the relationship between latitude & UVR/vitamin D and MS risk(16) & clinical course(17, 18), we sought to re-evaluate the geoepidemiology of MS prevalence using a meta-analysis study design.

3.3 Methods

3.3.1 Literature search

We searched PubMed (http://www.pubmed.org), EMBASE (http://www.embase.com), and ISI Web of Knowledge (http://www.isiknowledge.com) for articles matching the keywords "multiple sclerosis AND prevalence" or "multiple sclerosis AND epidemiology" for all publications which could be found up to publication-year 2010. In addition, article bibliographies were screened and some authors referred us to other prevalence studies.

3.3.2 Inclusion criteria

To be included, studies needed to have provided crude and/or age-specific prevalence-estimates with definition of the study area, source population and study period. Where this information was not reported, this information was sought from the study authors. The majority of scientific articles were published in English, but also included were articles written in Latin and Cyrillic-based alphabets. Articles were translated by the first author or using online translation software (http://translate.google.com).

3.3.3 Data-collection

The following information was abstracted from the study reports: study area, the study prevalence-year or final year of a period-prevalence study, the diagnostic criteria used, the source and study populations, and the crude and/or age-specific prevalence data.

3.3.4 HLA analysis

HLA-DRB1 allele frequencies for Europe were obtained from the online database http://www.allelefrequencies.net(19), or individual publications.

3.3.5 Statistical analysis

3.3.5.1 Crude prevalence

Crude prevalence was calculated as the number of prevalent cases ascertained in each study divided by the number of persons in the study population. Where the population size was not reported and was not available from local statistical sources, it was approximated from the reported prevalence-estimates and the reported number of cases. The variance of each prevalence-estimate was calculated using standard methods(20).

3.3.5.2 Age-standardisation

Where age-specific data were available, age-standardised prevalence was calculated by the direct method(20) using each of three standard populations: 2009 World, 2009 Australia and 2009 Europe(21). We found no meaningful differences using the different standard populations, and only those for the 2009 Europe population are reported. The variance of each age-standardised prevalence-estimate was calculated using standard methods(20).

3.3.5.3 Transformation and study weighting

The prevalence-estimates were transformed if necessary to reduce heteroskedasticity for regression analyses(22). For example, age-standardised prevalence-estimates were analysed on a logarithmic scale. Each prevalence-estimate was weighted by the inverse of its variance, with the variance of transformed estimates approximated using the Delta method.

3.3.5.4 Meta-regression

3.3.5.4.1 Heterogeneity

There was considerable between-study variance in the prevalence-estimates, as evidence by the REML estimate of between study variance, τ^2, Cochran's *Q*-statistic, the *I*-squared statistic. The results for global prevalence (τ^2=1.237, Q=3.1 x 10^8, p<0.0001, I^2=100%), global prevalence with age-specific data (τ^2=0.783, *Q*=6.3 x 10^7, p<0.0001, I^2=100%), and age-standardised global prevalence (τ^2=0.764, *Q*=4.1 x 10^5, p<0.0001, I^2=99.43%) were each inconsistent with a shared common effect size.

Because it was not reasonable to assume that all the heterogeneity could be explained by model covariates, random-effects meta-regression models were fitted using STATA/SE for Windows (Version 10.1; StataCorp LP College Station, TX USA).

3.3.5.4.2 Adjustment for covariates

Covariates were specified *a priori*, in keeping with our hypothesis that prevalence varies with latitude. Other covariates included prevalence-year, the diagnostic criteria used, and the inclusion of possible cases.

All regression models included adjustment for prevalence-year because, on average, the prevalence-estimates increased with time. Most models included a binary covariate for the type of diagnostic criteria used (1=Poser criteria and its variants, 2001 McDonald criteria or 2005 McDonald/Polman criteria, 0=all other diagnostic criteria or studies not specifying or not using systematic diagnostic criteria). In addition, some models included a binary covariate for inclusion of cases classified as possible MS (1=possible cases included, 0=possible cases not included). To improve fit to the data, some models included a product-term formed from the covariates for prevalence-year and diagnostic criteria, and a second product-term formed from the covariates for diagnostic criteria and possible cases. The estimates reported are those for the year 2009 and are calculated at the mean levels of the other covariates.

3.3.5.4.3 Time-corrected analysis

The prevalence-estimates depicted in Figure 3.2 for each study (the centres of the circles) are the predicted values from a regression model containing covariates for latitude and actual prevalence-year but calculated with prevalence-year set at 2009. They are estimates of the values that would have been obtained had each study been conducted in 2009.

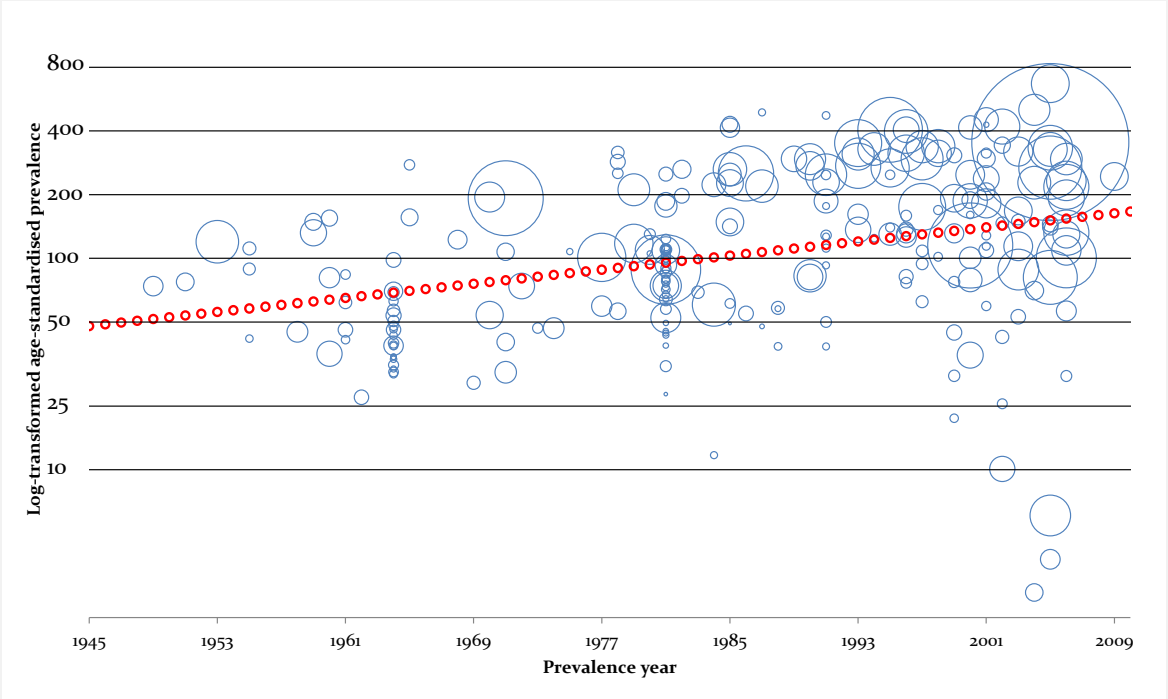
Methods note 3.1 Time-correction functions

A key enhancement of our study relative to those preceding is our adjustment for prevalence year. It is generally understood that prevalence will increase in serially-measured studies, a consequence of increasing incidence, influx of migrants from high-incidence regions, aging of the population, increased survival and/or increased case ascertainment (better infrastructure/access to care, better diagnostic

criteria). All of our prevalence estimates are age-standardised to a common population, removing the effects of changing population structure; however the other factors changing over time cannot be taken into account individually. Thus time itself need be taken into account. Other studies (Koch-Henriksen & Sørensen 2010) attempted to address this factor by only including the most recent study for serially-measured sites. However there is a general trend to an increase in prevalence over time (Methods note 3.1 Figure A), which is only slightly attenuated if serial measures are excluded (Methods note 3.1 Figure B).

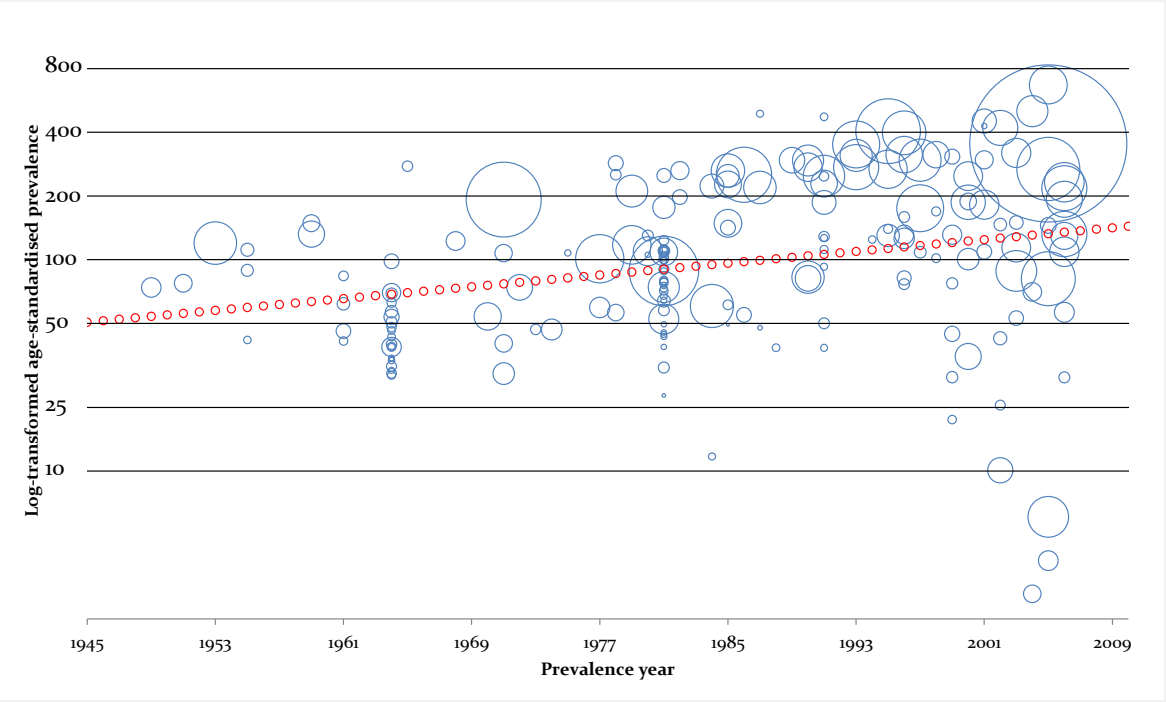
Methods note 3.1 Figure A. Log-transformed prevalence vs. prevalence year.

Size of bubbles proportionate to inverse of within-study variance. Red line corresponds to linear meta-regression function.



Methods note 3.1 Figure B. Log-transformed prevalence vs. prevalence year, excluding serial measures at same site.

Size of bubbles proportionate to inverse of within-study variance. Red line corresponds to linear meta-regression function.



We addressed this issue by adjusting all analyses where latitude was the primary predictor of interest for prevalence year. For graphical depictions of the time-adjusted prevalence against latitude, we estimated each prevalence at a common point in time, this chosen as the year 2009. This was done by meta-regression of the log-transformed prevalence estimate on predictor variables including a continuous term for latitude, a continuous term for prevalence year, a dichotomous term for whether the prevalence study used pre or post-Poser diagnostic criteria (*dxcrit*), a dichotomous term for whether the prevalence study included possible cases in calculating their prevalence estimates (*possible*), and an interaction term between the diagnostic criteria and possible case inclusion terms. From this, the residuals (difference between the estimated values from the regression function at each point and its actual reported value) were calculated and added to the predicted values from the regression function as:

$$Time-adjusted\ prevalence = \beta_0 + \beta_{latitude} x\ latitude + \beta_{year} x \text{year} + \beta_{dxcrit} x\ dxcrit + \beta_{possible} x\ possible + \beta_{dxcritxpossible} x\ dxcrit\ x\ possible + residual$$

where year was set equal to 2009.

3.3.5.4.4 Segmented analysis

Examination of the data revealed that the positive association between prevalence and latitude became less pronounced at high latitudes. To accommodate this, segmented models were fitted for supra-regions (global, Western Europe and Europe overall) that non-exclusively included areas located at high latitudes. The segmented models included a covariate for latitude when fitted for latitudes less than or equal to a threshold latitude (L_0), and covariates for latitude and its square when fitted for latitudes greater than the threshold. Reported in the text is the result of a test of the coefficient of the quadratic term. To estimate the threshold value, the segmented model was first estimated by weighted non-linear least squares minimisation using the PROC NLIN procedure in SAS (Version 9.2; SAS Institute Inc. Cary, NC USA). The estimated thresholds for the global model were $L_0 = 54 \cdot 4°$ (crude prevalence), $L_0 = 50 \cdot 7°$ (crude prevalence with age-specific data available) and $L_0 = 48 \cdot 8°$ (age-standardised prevalence).

3.3.5.4.5 Adjustment for HLA-DRB1

To assess the contribution of differences in population frequencies of several key MS-associated HLA-DRB1 alleles (HLA-DRB1*15, *11, *01, *03, and *14) to the latitudinal gradient within Europe, linear covariates were added for each allele.

3.3.5.4.6 Latitudinal gradient by sex

Sex-specific gradients in age-standardised prevalence with latitude were estimated in a model that included a binary covariate for sex (1 = females, 0 = males) and a product-term formed from the

covariates for latitude and sex. A statistical test of the coefficient of the product-term was used to compare the latitudinal gradients for males and females. Because the age-standardised prevalence-estimates had been log-transformed for analysis, this was equivalent to a test of whether the female-to-male ratio of age-standardised prevalence varied by latitude. A test of whether the female-to-male ratio of age-standardised prevalence varied by prevalence-year was conducted as a test of the coefficient of a product-term formed from the covariates for prevalence-year and sex.

3.4 Results

3.4.1 Review of literature

Literature searches using the keywords "multiple sclerosis AND prevalence" or "multiple sclerosis AND epidemiology" produced 9,379 and 14,808 results respectively. Additional studies were found by searching article references and from author referrals. A total of 365 studies, of which 321 were peer-reviewed, satisfied our inclusion criteria. Only the peer-reviewed studies were used in analyses unless otherwise specified. This provided 650 prevalence-estimates of which 239 could be age-standardised, and 159 of these included sex-specific data. The distribution of all prevalence estimates is depicted in Figure 3.1.

Figure 3.1. World map showing the distribution of all prevalence estimates included in this meta-analysis.

Summary information about the studies is shown in Table 3.1. More detailed information including study area, latitude and prevalence-year, diagnostic criteria, and prevalence estimates are shown in Appendix 3.1. Diagnostic criteria used in each study are outlined in Appendix 3.2. Rationales for allocation of study areas to study regions are described in Appendix 3.3. Data on HLA-DRB1 allele frequencies for each study area in Europe for which data could be obtained are shown in Appendix 3.4.

Table 3.1. Regional distribution of the 321 studies and their prevalence estimates.

Numbers in parentheses include non-peer-reviewed studies.

	Studies	Prevalence estimates	Age-standardised prevalence estimates	Sex-specific, age-standardised prevalence estimates
Australasia	16 (16)	31 (31)	27 (27)	26 (26)
Western Europe				
United Kingdom & Ireland	36 (40)	47 (54)	21 (24)	21 (24)
Scandinavia & North Atlantic	41 (41)	101 (102)	41 (41)	18 (18)
Atlantic & Central Europe	48 (57)	130 (143)	20 (20)	18 (18)
Italian region	55 (59)	66 (71)	31 (31)	30 (30)
Eastern Europe	39 (51)	144 (184)	48 (49)	16 (17)
North America	43 (47)	58 (62)	30 (30)	13 (13)
Latin America & Caribbean	2 (17)	21 (28)	4 (4)	4 (4)
Middle East & Africa*	16 (21)	20 (25)	11 (12)	9 (10)
Asia & Pacific	16 (16)	32 (32)	6 (6)	4 (4)
Total	**321 (365)**	**650 (732)**	**239 (244)**	**159 (164)**

Australasia (including Australia and New Zealand); **UK region** (including the United Kingdom of Greater Britain and Northern Ireland (England, Northern Ireland, Scotland, and Wales, the Republic of Ireland, and the Orkney Islands (UK); **Scandinavia & North Atlantic** (including Denmark, Finland, Iceland, Norway, Sweden, the Faroe Islands (Denmark), and the Shetland Islands (UK)), **Atlantic & Central Europe** (including Belgium, the Czech Republic, France, Germany, the Netherlands, Portugal, Spain (continental, the Balearic Islands and the Canary Islands), and Switzerland); **the Italian Region** (including Peninsular and Insular Italy, San Marino, and the island-région of Corsica of France); **Eastern Europe** (including Albania, Bosnia-Herzegovina, Bulgaria, Croatia, Estonia, Greece, Hungary, Lithuania, Macedonia, Poland, Romania, Russia, Serbia, Ukraine, and the country formerly known as Yugoslavia); **North America** (including Canada and continental & insular United States of America); **Latin America & the Caribbean** (including Argentina, Brazil, Chile, Colombia, Ecuador, Mexico, Panama, Peru, Uruguay and the French West Antilles); **the Middle East & Africa** (including Iran, Iraq, Israel, Jordan, Kuwait, Libya, Malta Qatar, Saudi Arabia, South Africa, and Turkey); **Asia & Pacific Islands**(including Fiji, India, Japan, the People's Republic of China, and the Republic of China (Taiwan)). * Note: The nation of Malta is allocated to the Middle East & Africa region (see Supplement 3 for rationale), however for analyses of Western Europe, Malta is included.

3.4.2 Global analyses

Prevalence was significantly (p=0.001) associated with latitude. Restricting the analysis to prevalence-estimates that could be age-standardised attenuated the association, but it remained statistically-significant (p=0.001) including after age-standardisation (p<0.001). On average, the prevalence-estimates increased with prevalence-year (p<0.001, data not shown). Adjusted for prevalence-year, the strength of the association between prevalence and latitude increased in all analyses. Further adjusting for diagnostic criteria and inclusion of possible cases slightly reduced the latitudinal gradient vis-à-vis adjustment for prevalence-year alone (Table 3.2).

Table 3.2. Estimated change in prevalence/100,000 per-degree-of-latitude showing the effect of adjustment for year of the study, use of systematic diagnostic criteria and inclusion of possible cases.

			Prevalence-estimates with age-specific data			
	All crude		Crude		Age-standardised	
	Slope	(95% CI) *	Slope	(95% CI) *	Slope	(95% CI) *
Unadjusted	1.58	(1.30, 1.87)†	0.81	(0.34, 1.28)†	1.04	(0.51, 1.56)†
Adj. for prevalence-year	3.92	(3.15, 4.70)†	2.64	(1.54, 3.74)†	2.94	(1.74, 4.15)†
Fully-adjusted model‡	3.32	(2.57, 4.07)†	2.30	(1.27, 3.33)†	2.60	(1.44, 3.77)†

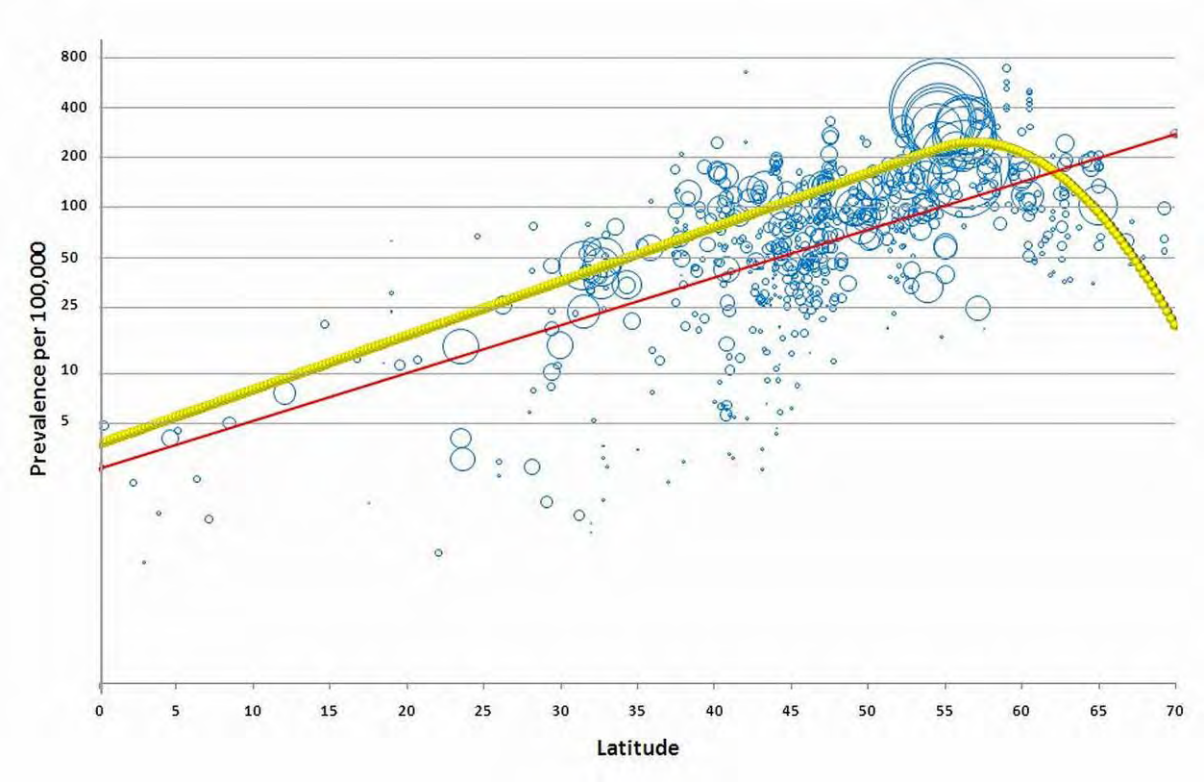
* Slope (95% CI) = change in prevalence/100,000 per-degree-of-latitude at the mean global latitude (46.1°) (95% confidence interval)
† Statistically-significant (p<0.001)
‡ Adjusted for prevalence-year, use of systematic diagnostic criteria and inclusion of possible cases.
Note The model estimates of the residual variance for each age-standardised prevalence model were: 0.7636 (model without covariates), 0.7146 (model with latitude), 0.5839 (model with latitude and prevalence-year), and 0.5422 (fully-adjusted model).

3.4.2.1 Models allowing a reducing gradient at high latitudes

A model that allowed additional covariates for latitude and its square to be fitted for high latitudes provided evidence of curvature that was statistically-significant (p<0.001) in each prevalence analysis (Figure 3.2).

Figure 3.2. Plot of time-corrected prevalence against latitude.

A) All crude prevalence-estimates; B) Crude prevalence-estimates restricted to those that could be age-standardised; C) Prevalence age-standardised to 2009 Europe population. The area of each circle is proportional to the inverse of the variance of the prevalence-estimates.



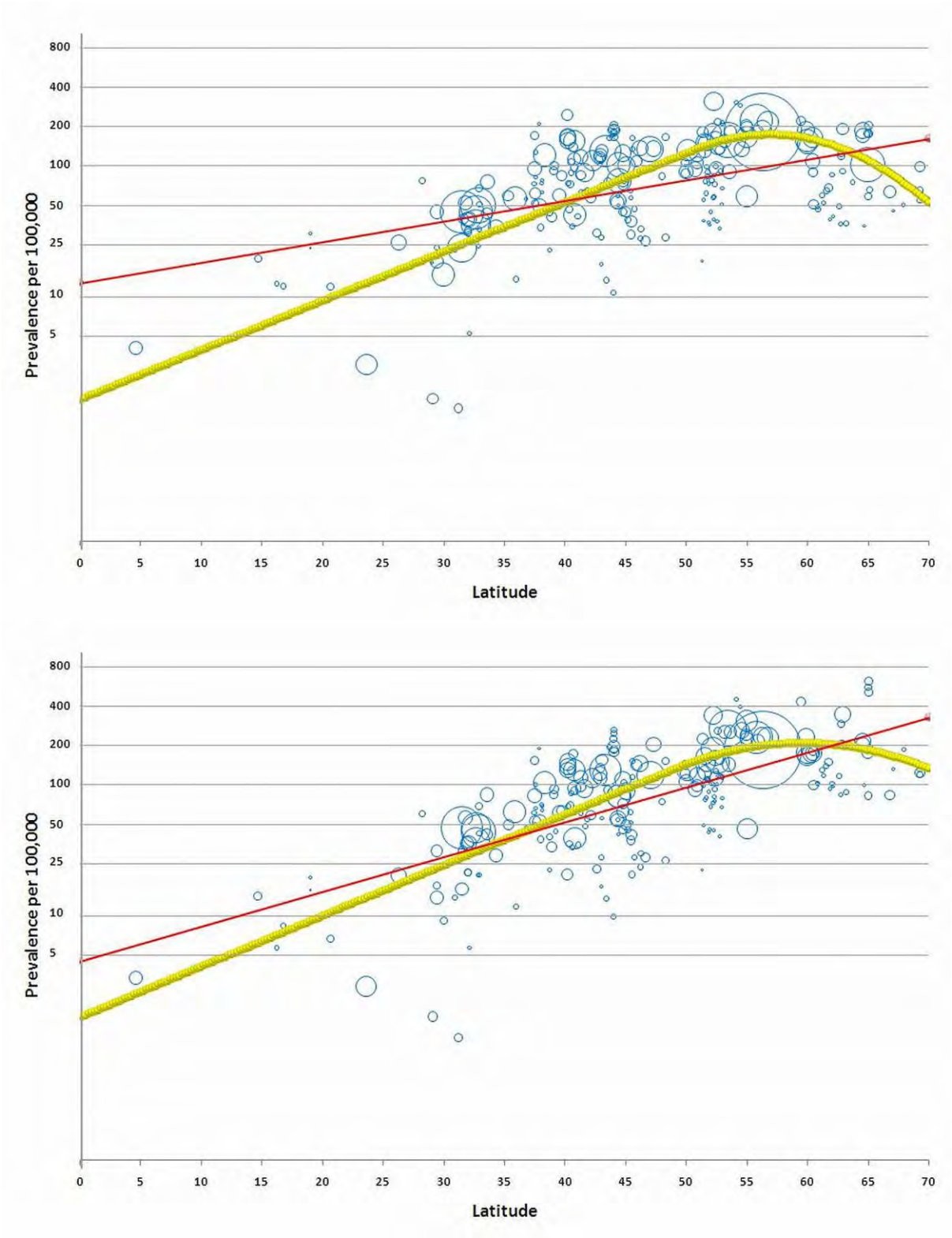


Table 3.3 shows the change in prevalence per degree latitude at five latitude degree increments for each of the analysis types. As in Table 3.2, the gradient is most potent when all prevalence are included; the gradient is moderated on restriction to crude prevalence with age-specific data, and enhanced on age-

standardisation. Also, similar to the trend lines in Figure 3.2, the gradient increases steadily with increasing latitude, reaching a peak around 55°, before changing to a significant inverse gradient above 60°.

Table 3.3. Estimated change in prevalence/100,000 per-degree-of-latitude at increments of latitude.

			Prevalence-estimates with age-specific data			
	All crude		Crude		Age-standardised	
Latitude	Slope	(95% CI) *	Slope	(95% CI) *	Slope	(95% CI) *
0	0.18	(0.13, 0.22)†				
5	0.25	(0.20, 0.31)†	0.47	(0.33, 0.62)†	0.51	(0.36, 0.67)†
10	0.36	(0.29, 0.43)†	0.60	(0.44, 0.77)†	0.66	(0.47, 0.84)†
15	0.51	(0.43, 0.60)†	0.77	(0.57, 0.98)†	0.84	(0.62, 1.06)†
20	0.73	(0.62, 0.85)†	0.99	(0.74, 1.24)†	1.07	(0.80, 1.35)†
25	1.04	(0.89, 1.20)†	1.27	(0.95, 1.60)†	1.37	(1.01, 1.73)†
30	1.49	(1.26, 1.71)†	1.63	(1.20, 2.07)†	1.75	(1.27, 2.23)†
35	2.12	(1.78, 2.46)†	2.09	(1.48, 2.70)†	2.24	(1.57, 2.90)†
40	3.02	(2.48, 3.56)†	2.68	(1.82, 3.54)†	2.86	(1.92, 3.80)†
45	4.31	(3.44, 5.18)†	3.43	(2.21, 4.65)†	3.66	(2.33, 4.99)†
50	6.14	(4.73, 7.55)†	4.40	(2.66, 6.14)†	4.67	(2.78, 6.56)†
55	9.61	(7.08, 12.14)†	6.13	(3.45, 8.81)†	6.51	(3.60, 9.42)†
60	-3.17	(-6.40, 0.05)	-0.94	(-2.82, 0.94)	-1.15	(-3.42, 1.13)
65	-12.09	(-15.75, -8.44)†	-7.25	(-10.90, -3.60)†	-7.87	(-12.07, -3.66)†
70	-9.25	(-11.59, -6.92)†	-8.34	(-11.21, -5.47)†	-8.90	(-12.01, -5.80)†

* Slope (95% CI) = change in prevalence/100,000 per-degree-of-latitude at the specified latitude (95% confidence interval)
† Statistically-significant ($p<0.001$)
All analyses adjusted for prevalence-year, use of systematic diagnostic criteria and inclusion of possible cases.
Note: Values at 0 latitude not calculated for crude and age-standardised prevalence with age-specific data as there were no prevalence at this latitude with age-specific data.

3.4.3 Regional analyses

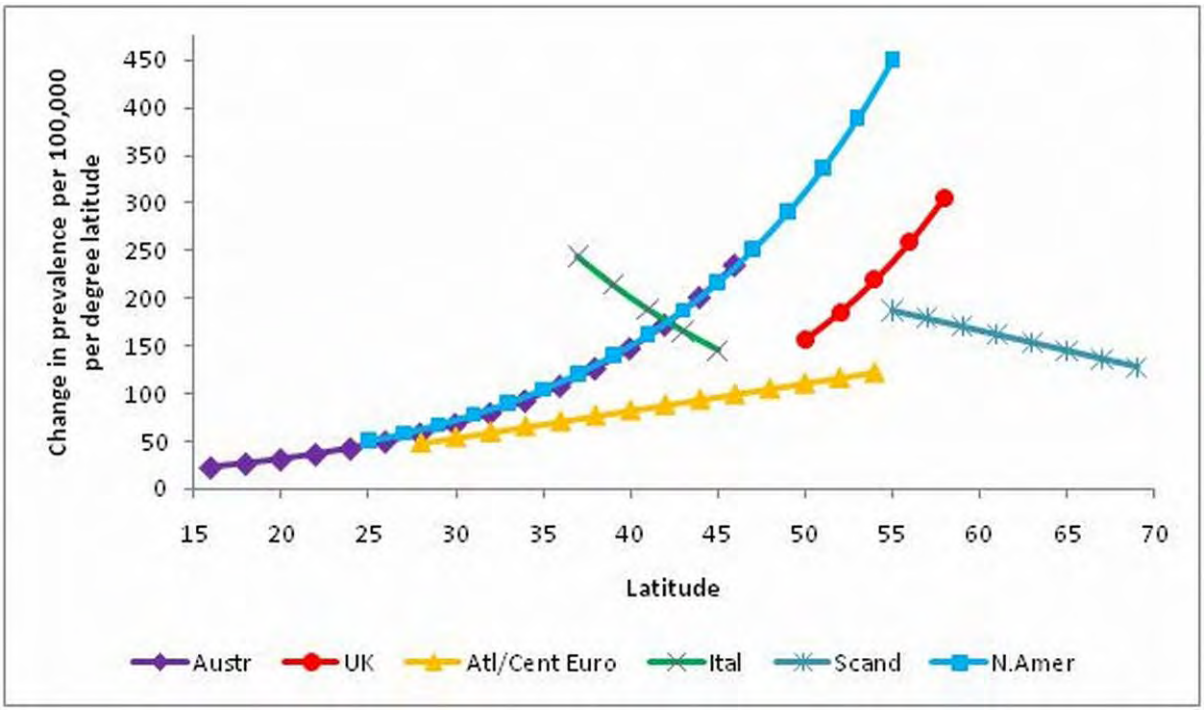
In regional analyses (Table 3.4, Figure 3.3), a statistically-significant positive gradient was found within Australasia, the UK region, Atlantic & Central Europe, North America, and Western Europe overall. A statistically-significant inverse gradient was found within the Scandinavia & North Atlantic and Italian regions.

Table 3.4. Region-specific associations between latitude and time-adjusted, age-standardised prevalence.

Region	Number of age-standardised prevalence estimates	Midpoint latitude	Slope	95% CI*
Australasia	27	35.51	8.38	(5.77, 10.98)††
Western Europe	114	50.75	8.11	(3.85, 12.35)†
UK region	21	54.64	19.81	(7.11, 32.51)†
Scandinavia & North Atlantic	41	61.25	-4.29	(-7.59, -0.99)†
Atlantic & Central Europe	20	46.25	2.82	(0.42, 5.21)†
Italian region	31	41.32	-11.59	(-20.17, -3.02)†
Eastern Europe	48	47.24	-0.76	(-4.67, 3.15)
North America	30	44.06	15.35	(6.37, 24.32)††
Latin America & the Caribbean	4	20.76	0.06	(-1.56, 1.68)
Middle East & Africa	11	31.83	1.62	(4.26, 7.50)
Asia & Pacific Islands	6	32.94	0.90	(-3.24, 5.03)

* Slope (95% CI) = change in prevalence/100,000 per degree of latitude (95% confidence interval)
† Statistically significant ($p<0.05$); †† Statistically significant ($p<0.001$)
Data for Australasia, Western Europe including the UK region, the Scandinavia & North Atlantic region, the Atlantic & Central Europe region, the Italian region, the Eastern Europe region, and Malta, North America, Latin America & the Caribbean, the Middle East & Africa region and the Asia & Pacific Islands region

Figure 3.3. Region-specific gradients per degree-latitude for Australasia, Western Europe, and North America.



For nations of largely European-descent (Europe, Australasia, North America, Latin America excluding the French West Antilles, and Israel), the latitudinal gradient in age-standardised prevalence was 3.97 (95% CI: 2.27, 5.66) cases/100,000 per-degree-of-latitude. For all other nations for which we had prevalence data, here defined as non-European descent, the latitudinal gradient was -0.07 (95% CI: -1.07, 0.93) cases/100,000 per-degree-of-latitude, and the difference in trend was statistically-significant (p=0.04).

3.4.4 Adjustment for HLA-DRB1

Table 3.5 shows the effects of adjustment for the frequencies of several HLA-DRB1 alleles on the gradients within Europe. The significant inverse gradient in the Italian region was completely reversed on adjustment, whilst the positive gradient for Western Europe was almost unchanged and that for Europe enhanced by 33.4 percent.

Table 3.5. Associations between latitude and time-adjusted age-standardised prevalence for the Italian, Western Europe and Europe regions, and with adjustment for HLA-DRB1[‡] frequencies

Region	Number of age-standardised prevalence-estimates	Slope	95% CI*
Italian region			
All prevalence-estimates	31	-11.59	(-20.17, -3.02)†
Adjusted for HLA-DRB1‡	31	5.99	(-22.94, 34.91)
Western Europe			
All prevalence-estimates	114	9.27	(4.23, 14.33)††
Only those with HLA-DRB1 data	99	7.70	(3.19, 12.22)††
Adjusted for HLA-DRB1‡	99	7.97	(3.11, 12.84)††
Europe			
All prevalence-estimates	162	5.57	(2.34, 8.80)††
Only those with HLA-DRB1 data	146	5.03	(1.61, 8.46)††
Adjusted for HLA-DRB1‡	146	6.71	(2.43, 10.98)††

* Slope (95% CI) = change in prevalence/100,000 per-degree-of-latitude (95% confidence interval)
† Statistically-significant (p<0.05); †† Statistically-significant (p<0.001)
‡Adjustment for HLA-DRB1 allele frequencies includes adjustment for HLA-DRB1*01, *03, *11, *14, & *15.

3.4.5 Latitudinal gradient by sex

The global latitudinal gradients in age-standardised prevalence for males and females were 4.09 (95% CI: 2.80, 5.39) and 7.19 (95% CI: 4.84, 9.53) cases/100,000 per-degree-of-latitude respectively, at the mean global latitude. These estimates were not statistically distinguishable (p=0.358), and hence there was not a statistically-significant change in the female/male ratio of age-standardised prevalence with latitude. At latitudes up to 59°, the prevalence sex ratio was 2.03 (95% CI: 1.71, 2.42) but without evidence of any significant change with latitude over this range (p=0.768); above latitude 59°, the prevalence sex ratio was 1.59 (95% CI: 1.25, 2.02), but again without any evidence of a significant change with latitude over this range (p=0.386).

The prevalence sex ratio did increase over time, increasing from 1.38 in 1949 to 2.34 in 2009, but this did not reach statistical-significance (p=0.12) in this sample size. Evaluating the change in prevalence sex ratio within regions found no significant change over latitude in any region; however there was a statistically significant increase in the prevalence sex ratio over time in Australasia (p=0.023) and the UK region (p=0.003).

3.4.6 Exclusion of serial measures

Serial measurements within one location – most commonly in high-prevalence areas of Europe, North America and Australasia – effectively re-sample the same population if closely-spaced in time. To evaluate potential bias, we restricted the analyses to the most recent prevalence-estimates for each location, finding this made no material difference to the results (data not shown).

3.4.7 Exclusion of non-systematic diagnostic criteria

Another potential source of bias was the inclusion of studies using non-systematic MS diagnostic criteria. Excluding these studies resulted in no material changes in the estimated associations between prevalence and latitude (data not shown).

3.4.8 Inclusion of non-peer reviewed studies

Excluded from all analyses thus far were studies (n=47) that were not peer-reviewed. Including them made no material difference (data not shown).

3.5 Discussion

This is the most comprehensive meta-analysis of MS prevalence studies yet undertaken, including 650 prevalence-estimates from 321 peer-reviewed studies in 59 countries between 1923 and 2009. We found a strong and statistically-significant latitudinal gradient for prevalence globally, which persisted on age-standardisation and was enhanced on adjustment for prevalence-year. The latitudinal gradient was observed only among nations of largely European-descent, and while the distribution of HLA-DRB1 alleles did not explain the positive gradient in Europe or Western Europe, adjustment for HLA-DRB1 allele distribution reversed the inverse gradient in the Italian region. Similar gradients were observed for males and females and the prevalence sex ratio did not change with latitude, or over time.

3.5.1 Exceptions to the gradient

3.5.1.1 European vs. non-European populations

That there was a significant association between latitude and prevalence for European-descent regions, which was absent for regions of largely non-European-descent, suggests the presence of gene-environment interactions. This is not unexpected, given the higher frequencies of high-risk alleles for MS in European populations(23). Interactions between the actual genes (e.g. HLA-DRB1*1501) and environmental risk factors (e.g. exposure to UVR) are likely to exist and the identification of those interactions is an emerging field or research. Moreover, other aspects such as epigenetic modifications and the timing of exposures further complicate the etiology of MS.

3.5.1.2 Italian region

In the Italian region, we observed a significant inverse gradient. On adjustment for all HLA-DRB1 allele frequencies, the inverse gradient was reversed, yielding a positive gradient similar to the rest of

Europe. This suggests that the inverse gradient in the region is entirely due to the unique distribution of HLA-DRB1 alleles in this area.

3.5.1.3 Scandinavia & North Atlantic

Due to a paucity of data on HLA-DRB1 allele frequencies by latitude in the Scandinavia region, we were unable to evaluate their role in this region. Populations in northern Scandinavia are a unique admixture of Swedes, Finns and Sámi(24). While none of the prevalence studies reported large proportions of low-risk groups like the Sámi in their source populations (11%(14, 25) to 12%(26)), it may be that ancestral components from the Sámi contribute to the lower prevalence at these latitudes.

A possible explanation for the inverse gradient in the region was suggested by Kampman & Brustad(27) While latitude correlates with reduced winter UVR and lower vitamin D, in Scandinavia higher latitude does not result in the low levels of serum vitamin D expected due to high dietary intake. Particularly at the northern latitudes(27-30), dietary consumption of vitamin D in Scandinavia far exceeds that of other European populations, particularly in winter: dietary intake in peninsular Scandinavia ranges from 6.0 to 9.9μg/day(29-31), while intake in continental Europe is lower, ranging from 2.0 to 3.3μg/day(32). Thus, despite the absence of vitamin D-generating UVR, mean serum vitamin D metabolite (25(OH)D) levels remain close to 50nmol/L during winter at latitudes up to 71°N(29, 31, 33). There is now substantial evidence that exposure to UVR or vitamin D is associated with MS onset(34, 35) and this increased dietary intake of vitamin D could contribute to the region's inverse gradient.

3.5.2 HLA-DRB1 & the gradient in Europe

Importantly, our analysis showed little effect on the latitudinal gradient in Europe after adjustment for the distribution of MS-associated HLA-DRB1 allele frequencies. This is in contradistinction to others(36) who found that the distribution of HLA-DRB1 accounted for 52% of the variation in prevalence by latitude in Europe, while the UVR index accounted for only 31% in univariable analysis. In our analyses we were able to assign HLA-DRB1 allele frequencies in Europe to a much finer degree

than attempted previously(36), finding that adjustment for HLA-DRB1 frequencies increased the latitudinal gradient in Western Europe by 3.5% and the gradient in Europe overall by 33.4%. These findings suggest a strong independent role for non-HLA-DRB1 factors in the gradient in Europe.

3.5.3 Sex and prevalence sex ratio

All trends observed in the total were mirrored in each sex, and no significant difference by sex was observed in any of our analyses. Globally, we found no significant change in the prevalence sex ratio (female/male) across the latitudinal range, nor within the intervals up to and above 59°. We found a 70% increase in the prevalence sex ratio over the 60-year interval for which we have prevalence data, but this did not reach statistical significance in this sample size. These results are different from those reported elsewhere(6), and may reflect different methods and data included. On examining changes within regions, in no instance did we find a significant change in the prevalence sex ratio over latitude. In some regions we found an increase in the prevalence sex ratio over time, including significant increases in Australasia and the UK region, while in other regions such as North America, an increase was found but did not reach statistical significance. These regional findings are somewhat in conflict with the significantly increasing prevalence sex ratio over latitude in New Zealand(8) and the significantly increasing prevalence sex ratio over time in Canada(37). This disparity may reflect less comprehensive coverage of these regions in our analysis, since a minority of studies provided age and sex-specific prevalence data.

3.5.4 Strengths and improvements from previous studies

This study makes significant improvements upon previous meta-analyses by Koch-Henriksen & Sørensen(6) and Zivadinov(4), and preceding descriptive reviews(1-3), in a number of key elements. These methodological improvements, both in data-collection and statistical analysis, provide strong support in favour of our conclusions, and no doubt explain the differential findings from previous studies.

At the most basic, our study is more comprehensive, encompassing a broader range of studies, both geographical and temporal, that satisfy the inclusion criteria. This is due to our use of multiple data sources, as well as our inclusion of studies published in languages other than English, allowing a more powerful evaluation of geographic and temporal changes in prevalence.

Our study improves upon the work of Zivadinov(4) in our use of study weighting by the inverse of study variance. As in Figure 3.2 of our paper, there are a number of small outliers, particularly in the crude analysis. If these studies are not weighted proportionate to their small size and high variance, they can potentially affect the interpretation of the associations measured. The study by Koch-Henriksen & Sørensen(6) restricted their inclusion of studies, requiring a minimum of 20 cases. While this is one method of addressing variable study quality, this has the effect of moderating any potential gradient, since the number of cases, presuming a constant population, would decrease with decreasing latitude; removing studies with smaller prevalent cohorts would remove a greater proportion of studies at the lower extreme of latitude, biasing the results. Rather as we have done, the use of study weighting by the inverse of study variance would address any potential differences in study quality which might co-vary with case number, while preserving the maximal study inclusiveness.

A key feature of our study relative to most others(1-3, 6) is the use of age-standardisation. As noted by Zivadinov(4), age-standardisation is requisite for studies comparing aggregate-level data from different study regions, which can have significantly different age-structures. While some have suggested that age-standardisation was unnecessary(6), it made an important difference to the findings of Zivadinov(4), Alonso & Hernán(5), and our own.

Our analyses were strengthened by adjustment for prevalence-year, which was significantly associated with prevalence independently of other covariates. Importantly, we observed an association between log-transformed prevalence and latitude prior to time-adjustment, which was enhanced on simple adjustment for prevalence year, indicating that our findings are not a statistical artifact of the time-adjustment process. Interestingly, Koch-Henriksen & Sørensen(6) also find a significant association between prevalence and prevalence year, however they do not adjust their analyses of prevalence and latitude for it – doing so in our analysis significantly enhanced the magnitude and significance of the association between latitude and prevalence. Our results show that failure to adjust for prevalence-year would underestimate the magnitude and significance of the latitudinal gradient (Table 3.2), particularly when the meta-analysis includes studies over a wide range of time, as was the case here.

A novel feature of our analysis is the segmented, rather than simple linear models used to evaluate the global gradient: the prevalence gradient increased with latitude, reaching a peak around 55°, before becoming a significant inverse gradient above 60° (Figure 3.2, Table 3.3). This reduction in the gradient at higher latitudes was also observed by Zivadinov and colleagues(4); their use of a linear trend to evaluate the significance of the global gradient may contribute to their conclusion of an attenuation after age-standardisation, rather than an enhancement as observed here.

For our HLA-DRB1 analyses, we were able to ascribe HLA-DRB1 allele frequencies with a much greater precision than attempted previously. Whereas previous studies(36) have evaluated the relationship between prevalence, latitude and HLA-DRB1 at the national or supra-local level, we were able to assign HLA-DRB1 allele frequencies to the majority of the individual prevalence-estimates in Europe using HLA-DRB1 surveys within geographically-relevant areas. In an area of such genetic complexity as Europe, this is critical in evaluating the role of HLA-DRB1 in the latitudinal gradient.

3.5.5 Study weakness

Several weaknesses of this study need to be borne in mind. Firstly, the analyses are based on prevalence-estimates made in different study centres with varying degrees of case-ascertainment and different study procedures. Our inclusion criteria excluded case-series and other non-population-based estimates of prevalence. However, we did not attempt to grade and select studies for inclusion based on perceived study quality, because to do so requires objective information that is rarely fully-reported in MS prevalence studies. Instead, we attempted to take some of the known factors into account in analyses; however we accept that it is not possible to do so completely.

We were not able to remove all residual between-study variance using information available to use on pre-specified covariates. This residual variance was most pronounced at the global level; however in regional analyses (data not shown) the covariates were successful in explaining a much larger part of the between-study variance. This added to confidence that the association of MS prevalence with model covariates – including latitude – is truly reflective of causal factors that correlate with latitude, most particularly environmental factors like UVR/vitamin D.

There are potential sources of bias in our own study procedures, including selection bias from inclusion of serially-measured prevalence-estimates at the same location or exclusion of non-peer-reviewed studies, and measurement bias from including studies making use of non-systematic diagnostic criteria. However, none of these factors had a material impact on our findings.

A further issue is that of publication bias. We have attempted to address publication bias by drawing our studies from a broad a range of sources, and including studies published in languages other than English, as well as including non-peer-reviewed studies in a sub-analysis. Publication bias on the part of the individual study authors is less of a concern than in some other study types, because prevalence

studies are necessarily less prone to publication bias by virtue of the absence of a "null finding". Furthermore, if authors do not pursue publication of findings that are not in marked contrast with previously reported estimates for their area, the published estimates nevertheless capture the regional variance and the non-published findings would not materially change our conclusions.

3.5.6 Conclusion

We here present the largest and most comprehensive study of MS prevalence yet done, finding a significant positive association between latitude and prevalence at the global level, as well as in most regions of European-descent. Our findings are inconsistent with preceding reviews of MS prevalence(3, 4, 6), but in harmony with a methodologically similar meta-analysis of MS incidence(5). Our findings do not concur with all of the conclusions of previous meta analyses(4, 6) ; however we feel that the differences are accounted for by the improved methodologies as described. We feel that the inclusiveness and methodological improvements, particularly age standardisation and time adjustment, indicate that these findings are more representative of the current geoepidemiology of MS than previous studies. European exceptions to the gradient in the Scandinavia & North Atlantic and Italian regions are explicable by behavioural-cultural and genetic factors that vary geographically within these regions.

In contradistinction to work elsewhere(36). HLA-DRB1 variation did not account for the majority of the gradient in Europe, suggesting a greater role for environmental factors that vary by latitude, with the most prominent candidates being UVR and vitamin D. No doubt genetic and environmental factors interact to manifest in the variation in MS prevalence observed, but there are insufficient data available on the distribution of HLA-DRB1 alleles to quantify the proportions precisely. The information gleaned from this demonstration of the existence of a latitudinal gradient for MS prevalence will further the understanding of factors leading to MS and, potentially, help lead to its resolution.

3.6 Summary

Background: There is a striking latitudinal gradient in multiple sclerosis (MS) prevalence, but exceptions in Mediterranean Europe and northern Scandinavia, and some systematic reviews, have suggested that the gradient may be an artifact. We sought to evaluate the association between MS prevalence and latitude by meta-regression.

Methods & Findings: Studies sourced from online databases, reference mining and author referral. Prevalence estimates age-standardised to 2009 Europe population. Analyses by random-effects meta-regression, weighted with inverse of within-study variance. We included 650 prevalence estimates from 321 peer-reviewed studies; 239 could be age-standardised, 159 provided sex-specific data. We found a significant positive association (change in prevalence per degree-latitude) between age-standardised prevalence (1.04, $p<0.001$) and latitude that diminished at high latitudes. Adjustment for prevalence-year strengthened the association with latitude (2.60, $p<0.001$). An inverse gradient in the Italian region reversed on adjustment for MS-associated HLA-DRB1 allele distributions. Adjustment for HLA-DRB1 allele frequencies did not appreciably alter the gradient in Europe. Adjustment for some potential sources of bias did not affect the observed associations.

Conclusion: This, the most comprehensive review of MS prevalence to-date, has confirmed a statistically significant positive association between MS prevalence and latitude globally. Exceptions to the gradient in the Italian region and northern Scandinavia are likely a result of genetic and behavioural-cultural variations. The persistence of a positive gradient in Europe after adjustment for HLA-DRB1 allele frequencies strongly support a role for environmental factors which vary with latitude, the most prominent candidates being UVR/vitamin D.

3.7 Postscript

In this analysis I evaluated the largest and most comprehensive collection of MS prevalence data yet undertaken. By applying enhanced statistical analysis, most particularly the use of age-standardisation, study weighting and especially our use of time adjustment, I have conclusively demonstrated that a significant positive association exists between MS prevalence and latitude. This gradient exists at the global level, but even stronger gradients exist in Australasia, the UK and North America. This, in combination with the absence of any gradient among persons of non-European descent, indicates a significant interaction between the environmental factors, such as UVR and vitamin D, and genetic factors which vary between populations. On analyzing the exceptions to the gradient, in Northern Scandinavia and Mediterranean Europe, I found that there do exist idiosyncratic inverse gradients with latitude; however these may each be explained by genetic and behavioural-cultural features novel to these regions, and can be reconciled within the global positive gradient.

These results then provide strong support in favour of a role for factors that vary by latitude, particularly UVR and vitamin D, in mediating MS risk. These findings are in sync with case-control and cohort studies, which demonstrate significant inverse associations between personal UVR exposure and vitamin D levels and risk of MS.

3.8 References

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Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Data for each prevalence study, including study area and latitude, prevalence year, diagnostic criteria used, and crude and age-standardised prevalence data for total and by sex. Studies sorted by region, latitude and prevalence-year. Shaded rows correspond to study sub-populations; only rows in white were used in current analysis. † Includes possible cases

						Prevalence					
						Total		Males		Females	
Study area	Year	Latitude	Diagnostic criteria	Peer Rev.	Cases/ Population	Crude	Euro-Std	Crude	Euro-Std	Crude	Euro-Std
Appendix 3A.1 Australasia											
North Queensland[1], Commonwealth of Australia(1)	1981	16.75	Rose(2)	Yes	48†/ 433,052	11.08	15.45	6.23	9.04	16.31	21.40
Northern Territory, Commonwealth of Australia: Europeans only(3)	1960	18.50	Phys-diag	Yes	2†/ 21,053	9.50					
State of Queensland, Commonwealth of Australia(3)	1960	19.50	WFN(4)	Yes	128†/ 1,406,593	9.10					
South Queensland[2], Commonwealth of Australia(1)	1981	26.25	Rose(2)	Yes	356†/ 1,699,677	20.95	26.17	12.69	16.00	29.07	35.64
Rural Western Australia, Commonwealth of Australia(5)	1964	30.92	Allison & Millar(6)	Yes	39†/ 261,904	14.89	15.65	7.95	9.05	22.66	21.79
Perth, WA, Commonwealth of Australia(5, 7)	1961	31.95	Allison & Millar(6)	Yes	83†/ 420,133	19.76	22.99	12.68	14.83	26.51	30.58
Perth, WA, Commonwealth of Australia(8)	1981	31.95	Rose(2)	Yes	264†/ 898,918	29.37	37.12	15.36	20.47	42.98	52.61
State of South Australia, Commonwealth of Australia(9)	1961	32.03	Allison & Millar(6)	Yes	351†/ 988,732	35.50					
State of South Australia, Commonwealth of Australia UK-born only(9)	1961	32.03	Allison & Millar(6)	Yes	30†/ 78,786	38.08					
State of South Australia, Commonwealth of Australia Continental Europe-born only(9)	1961	32.03	Allison & Millar(6)	Yes	17/ 97,000	17.53					
State of South Australia, Commonwealth of Australia(10)	1981	32.03	Rose(2)	Yes	378†/ 1,285,714	29.40	37.09	17.80	22.67	40.80	50.51
State of New South Wales, Commonwealth of Australia(10)	1981	32.83	Rose(2)	Yes	1,907†/ 5,126,344	37.20	44.24	22.60	27.27	51.60	60.04
Newcastle, NSW, Commonwealth of Australia(7)	1961	32.92	Allison & Millar(6)	Yes	28†/ 142,574	19.64	20.61	18.30	19.56	20.97	21.59
Newcastle, NSW, Commonwealth of Australia(8)	1981	32.92	Rose(2)	Yes	51†/ 135,177	37.73	42.83	25.55	29.19	49.54	55.52
Newcastle, NSW, Commonwealth of Australia(11)	1996	32.92	Rose(2)	Yes	79†/ 133,672	59.10	68.63	33.70	37.14	83.70	97.93
Australian Capital Territory, Commonwealth of Australia(12)	1996	35.30	Rose(2)	Yes	176†/ 308,025	57.14	63.00	32.21	36.19	82.46	87.95
Northland/Auckland census region, New Zealand(13, 14)	2006	35.85	Polman[3](15)	Yes	819/ 1,452,162	56.40	66.05	27.41	31.15	83.95	98.52
Bay of Plenty, New Zealand(16)	2001	37.50	Chancellor[4](16)	Yes	86/ 171,147	50.25	54.53	19.28	21.29	79.42	85.45
Bay of Plenty/Waikato/Gisborne census region, New Zealand(13, 14)	2006	37.89	Polman[3](15)	Yes	334/ 684,582	48.79	54.49	27.22	30.42	69.38	76.90
Waikato region, New Zealand(17)	1981	37.97	Alter[5](18, 19)	Yes	65/ 275,424	23.60	31.96	12.90	17.47	34.40	45.45
Manawatu-Wanganui/Tarnaki/ Hawkes Bay census region, New Zealand(13, 14)	2006	39.52	Polman[3](15)	Yes	279/ 474,330	58.82	64.74	25.51	27.80	90.51	99.11
Wellington, New Zealand(20)	1968	41.29	Hornabook(20)	Yes	139/ 296,156	46.93	61.23	27.64	35.61	66.29	85.08

[1] North Queensland defined as the State of Queensland north of 37°S
[2] South Queensland defined as the State of Queensland south of 37°S
[3] Polman criteria otherwise known as 2005 McDonald criteria
[4] The Chancellor criteria are virtually identical to the McDonald criteria, which were published at the time this prevalence study was being undertaken. The authors acknowledge the similarity but regard their criteria as unique.
[5] Electrophysiological paraclinical evidence not included in diagnosis

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Wellington, New Zealand(21, 22)	1983	41.29	McDonald & Halliday(23)	Yes	237†/ 341,454	69.41					
Wellington, New Zealand: Europeans only(21)	1983	41.29	McDonald & Halliday(23)	Yes	235†/ 293,287	80.13					
Tasman/Nelson/Marlborough/ Wellington census region, New Zealand(13, 14)	2006	41.58	Polman[3](15)	Yes	505/ 579,033	87.21	96.50	46.49	50.70	125.83	139.12
West Coast/Canterbury census region, New Zealand(13, 14)	2006	42.26	Polman[3](15)	Yes	598/ 553,158	108.11	116.11	57.65	60.95	156.43	167.43
Hobart, TAS, Commonwealth of Australia(7)	1961	42.88	Allison & Millar(6)	Yes	37†/ 115,932	31.92	41.92	19.18	23.22	44.37	59.31
Hobart, TAS, Commonwealth of Australia(8)	1981	42.88	Rose(2)	Yes	125†/ 168,361	74.25	92.84	51.98	62.22	95.75	121.34
Hobart, TAS, Commonwealth of Australia(24)	2001	42.88	Poser(25)	Yes	229/ 207,401	110.41	119.13	72.79	78.23	146.83	157.19
Hobart, TAS, Commonwealth of Australia(24)	2009	42.88	McDonald[6](26)	Yes	265/ 218,016	121.55	121.93	66.85	65.74	174.75	174.22
Christchurch, New Zealand(27)	1971	43.53	Allison & Millar(6)	Yes	116†/ 278,368	41.67	53.72				
Southland/Otago census region, New Zealand(17)	1981	45.88	Alter(18, 19)	Yes	195/ 284,672	68.50	88.41	33.80	43.48	103.00	130.22
Southland/Otago census region, New Zealand(13, 14)	2006	46.15	Polman[3](15)	Yes	382/ 284,679	134.19	147.28	68.61	72.41	197.56	216.95

Appendix 3A.2 United Kingdom of Great Britain & Northern Ireland and Republic of Ireland

Bailiwick of Guernsey, UK(28)	1991	49.50	Poser(25)	Yes	45/ 61,164	73.57					
Bailiwick of Jersey, UK(28)	1991	49.50	Poser(25)	Yes	84/ 84,082	99.90					
Brighton & Mid-Downs Health Districts, England, UK(29)	1991	50.08	Poser(25)	Yes	665/ 596,594	111.00	124.23	66.00	74.29	154.00	170.70
Cornwall, England, UK(30)	1958	50.30	Allison & Millar(6)	Yes	214/ 339,144	63.10					
Devon, England, UK(31)	2001	50.70	McDonald[6](26)	Yes	325/ 371,900	87.39	90.96	49.61	51.17	123.36	127.98
Southampton/ Southwest Hampshire Health Authority, England, UK(32)	1987	50.90	Allison & Millar(6)/ Poser(25)	Yes	411†/ 417,000	99.00	109.74	64.00	72.28	132.00	144.59
Sutton borough, South London, England, UK(33)	1985	51.33	Allison & Millar(6)	Yes	195†/ 169,565	115.00	124.53	74.20	80.15	152.00	165.82
Cardiff unitary authority, Wales, UK(34-36)	1985	51.50	Allison & Millar(6)/ Poser(25)	Yes	380/ 376,718	101.10	113.40	65.40	75.66	133.20	148.52
Cardiff unitary authority, Wales, UK(34)	2005	51.50	Poser(25)/ McDonald[6](26)	Yes	620/ 424,633	146.00	163.57	89.10	99.27	198.20	223.39
South Glamorgan County, Wales, UK(35)	1985	51.67	Poser(25)	Yes	441†/ 376,718	117.00	131.76	79.60	91.94	150.60	168.80
England & Wales, UK(37, 38)	1991	52.06	Phys-diag	No	3,677/ 3,618,690	101.61	112.23	63.60	67.12	136.90	154.20
South Cambridgeshire, England, UK(39)	1993	52.19	Poser(25)	Yes	441/ 289,500	152.33	175.23	80.61	93.27	223.52	251.50
Cambridge Health District, England, UK(40)	1990	52.38	Poser(25)	Yes	374†/ 288,410	130.00	147.03	75.00	84.26	184.00	205.43
Suffolk County, England, UK(41)	1988	52.43	Allison & Millar(6)	Yes	62†/ 31,379	197.58					
North Cambridgeshire, England, UK(42)	1993	52.58	Poser(25)	Yes	449/ 379,425	118.34	136.79	76.26	86.68	159.22	183.42
County Wexford, Republic of Ireland(43, 44)	2001	52.77	Poser(25)	Yes	126/ 104,391	120.70	148.28	88.00	107.92	154.00	185.85
County Wexford Republic of Ireland(45, 46)	2006	52.77	Polman[3](15)	No	329/ 113,347	290.26					
Bradford, England, UK(47)	2008	52.80	McDonald[6](26)	Yes	334/ 388,512	334.00					
Bradford, England, UK: Non-South Asians (Whites)(47)	2008	52.80	McDonald[6](26)	Yes	297/ 265,179	112.00					
Bradford, England, UK: South Asians(47)	2008	52.80	McDonald[6](26)	Yes	37/ 123,333	30.00					
Carlisle, England, UK(48)	1961	52.89	Poskanzer(49)	Yes	57†/ 71,072	80.20		56.00		104.90	

[6] McDonald criteria refer to the 2001 McDonald criteria

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Bassetlaw Health District, England, UK(50, 51)	1988	53.00	Phys-diag	Yes	98/ 100,000	98.00					
County South Dublin Republic of Ireland(45, 46)	2006	53.26	Polman[3](15)	No	130/ 101,721	127.80					
Republic of Ireland(52)	1971	53.43	Phys-diag	Yes	1,951/ 2,669,217	73.09	96.13	61.19	80.95	85.10	110.25
Rochdale, Manchester, England, UK(53)	1989	53.62	Allison & Millar(6)/ Poser(25)	Yes	254†/ 207,600	122.35	147.62	83.33	98.01	165.23	193.78
Leeds Health Authority, England, UK(54)	1996	53.80	Poser(25)	Yes	617/ 734,524	84.00					
Leeds Health Authority, England, UK(55)	1999	53.80	Poser(25)	Yes	680/ 728,832	93.30					
Isle of Man, UK(56)	2007	54.26	Phys-diag	No	114/ 80,058	142.40	143.12	83.50	80.53	199.83	201.37
Northeast Ireland, Republic of Ireland(57)	2004	54.58	Poser(25)/ McDonald[6](26)	Yes	370/ 160,451	230.60	251.80	157.00	168.94	300.80	328.91
Durham County, Scotland, UK(49)	1958	54.70	Poskanzer(49)	Yes	615†/ 1,497,000	41.08					
County Donegal, Republic of Ireland(43)	2001	54.92	Poser(25)	Yes	240/ 130,011	184.60	226.01	85.00	106.89	282.00	336.85
County Donegal Republic of Ireland(45, 46)	2006	54.92	Polman[3](15)	No	173/ 119,442	144.84					
Northern Ireland, UK(6)	1953	55.00	Allison & Millar(6)	Yes	700†/ 1,370,709	51.07	59.88	0.00	56.48	0.00	63.05
Northern Ireland, UK(58)	1986	55.00	Phys-diag	No	118/ 86,131	137.00					
Northern Ireland, UK(59)	1996	55.00	Poser(25)	Yes	254/ 151,011	168.20	202.66	104.10	129.02	229.90	271.17
Northumberland County, Scotland, UK(49)	1958	55.34	Poskanzer(49)	Yes	391†/ 811,400	44.36					
Lothian & Border Health Board Regions, Scotland, UK(60)	1995	55.75	Poser(25)	Yes	1,613/ 862,678	187.00	202.34	112.00	122.36	257.00	276.75
Glasgow, Scotland, UK(61)	1998	55.86	Phys-diag	Yes	245/ 169,000	144.97	156.79	72.00	77.40	213.00	230.67
Fife, Scotland, UK(62)	1996	56.22	Phys-diag	Yes	508/ 355,245	143.00	156.82	85.00	93.94	199.00	215.33
Tayside Health Board, Scotland, UK(63)	1996	56.70	Poser(25)	Yes	727/ 395,109	184.00	198.93	100.00	108.82	262.00	282.79
Northeast Scotland, UK(64)	1970	57.22	Alter(18, 19)	Yes	559†/ 437,737	127.00					
Northeast Scotland, UK(65)	1973	57.22	Alter(18, 19)	Yes	634†/ 441,880	144.00					
Northeast Scotland, UK(66)	1980	57.22	Alter(18, 19)/ Phadke & Downie[7](66)	Yes	839†/ 470,345	178.00					
County of Inverness, Scotland, UK(67)	1961	57.47	Sutherland(67)	Yes	47†/ 84,924	55.34					
County of Nairn, Scotland, UK(67)	1961	57.58	Sutherland(67)	Yes	4†/ 8,719	45.88					
Ross & Cromarty County, Scotland, UK(67)	1961	57.66	Sutherland(67)	Yes	26†/ 60,503	42.97					
Outer Hebrides, Scotland, UK(67, 68)	1954	57.72	Phys-diag	Yes	11†/ 35,807	30.72					
Outer Hebrides, Scotland, UK(68)	1979	57.72	Phys-diag	Yes	30†/ 30,844	97.26					
Scotland, UK(37, 38)	1992	57.72	Phys-diag	No	736/ 527,599	139.50	286.07	75.90	81.29	199.20	211.69
County of Sutherland, Scotland, UK(67)	1961	58.25	Sutherland(67)	Yes	9†/ 13,664	65.87					
County of Caithness, Scotland, UK(67)	1961	58.42	Sutherland(67)	Yes	20†/ 22,705	88.09					
Orkney Islands, Scotland, UK(67, 68)	1954	59.00	Sutherland(67)	Yes	23†/ 20,747	110.87					
Orkney Islands, Scotland, UK(68, 69)	1962	59.00	WFN(4)	Yes	33†/ 18,531	178.08					
Orkney Islands, Scotland, UK(68, 70)	1970	59.00	Allison & Millar(6)	Yes	40†/ 17,137	233.41					
Orkney Islands, Scotland, UK(68, 70)	1974	59.00	Allison & Millar(6)	Yes	54‡/ 17,462	309.25					
Orkney Islands, Scotland, UK(71)	1983	59.00	Poskanzer(49)	Yes	43/ 19,196	224.00					

[7] Phadke & Downie consolidate the early-probable and probable categories of the Alter criteria

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Appendix 3A.3 Scandinavia and North Atlantic

Malmöhus län, Kingdom of Sweden(72)	1934	55.78	Phys-diag	Yes	122/ 510,664	23.89					
Kristianstadt län, Kingdom of Sweden(72)	1934	55.90	Phys-diag	Yes	45/ 245,912	18.30					
Blekinge län, Kingdom of Sweden(72)	1934	56.24	Phys-diag	Yes	22/ 144,841	15.19					
Kingdom of Denmark(73)	1950	56.27	DMSR[8](73)	Yes	3,727/ 4,283,908	87.00					
Kingdom of Denmark(73)	1955	56.27	DMSR[8](73)	Yes	4,337/ 4,425,510	98.00					
Kingdom of Denmark(73)	1960	56.27	DMSR[8](73)	Yes	4,660/ 4,568,627	102.00					
Kingdom of Denmark(73)	1965	56.27	DMSR[8](73)	Yes	4,814/ 4,719,608	102.00					
Kingdom of Denmark(74)	1996	56.27	Poser(25)	Yes	6,445/ 5,261,224	122.50		89.40		154.80	
Kingdom of Denmark(75)	2005	56.27	Allison & Millar(6)/ Poser[9](25)	Yes	9,377/ 5,411,405	173.28	177.26				
Hallands län, Kingdom of Sweden(72)	1934	56.77	Phys-diag	Yes	29/ 150,128	19.32					
Kronobergs län, Kingdom of Sweden(72)	1934	56.78	Phys-diag	Yes	41/ 155,535	26.36					
Kalmar län, Kingdom of Sweden(72)	1934	57.19	Phys-diag	Yes	58/ 231,551	25.13					
Jönköpings län, Kingdom of Sweden(72)	1934	57.53	Phys-diag	Yes	63/ 231,557	27.21					
Gotlands län, Kingdom of Sweden(72)	1934	57.54	Phys-diag	Yes	2/ 57,448	3.48					
Göteborg, Kingdom of Sweden(76)	1978	57.70	Poser[101112](25)	Yes	399†/ 438,462	91.00					
Göteborg, Kingdom of Sweden(76)	1982	57.70	Poser[101112](25)	Yes	398†/ 437,363	91.00					
Göteborg, Kingdom of Sweden(76)	1988	57.70	Poser[1011](25)	Yes	415†/ 432,292	96.00					
Älvsborgs län, Kingdom of Sweden(72)	1934	58.17	Phys-diag	Yes	80/ 313,199	25.54					
Älvsborgs län, Kingdom of Sweden(77)	1966	58.17	Gilland[13](78)	Yes	280†/ 385,144	72.70					
Göteborgs och Bohus län, Kingdom of Sweden(72)	1934	58.21	Phys-diag	Yes	85/ 457,067	18.60					
Östergötlands län, Kingdom of Sweden(72)	1934	58.33	Phys-diag	Yes	69/ 309,995	22.26					
Skaraborgs län, Kingdom of Sweden(72)	1934	58.34	Phys-diag	Yes	76/ 242,329	31.36					
Södermanlands län, Kingdom of Sweden(72)	1934	59.11	Phys-diag	Yes	35/ 189,182	18.50					
Örebro län, Kingdom of Sweden(72)	1934	59.42	Phys-diag	Yes	64/ 219,236	29.19					
Vestfold fylke, Kingdom of Norway(79, 80)	1959	59.43	WFN(4)	Yes	127†/ 182,746	69.50	74.41	59.20	65.62	79.85	82.60
Vestfold fylke, Kingdom of Norway(81)	1963	59.43	McAlpine(82, 83)	Yes	105/ 177,541	59.14					
Vestfold fylke, Kingdom of Norway(81)	1983	59.43	McAlpine(82, 83)	Yes	163/ 188,864	86.40					
Stockholms län, Kingdom of Sweden(72)	1934	59.50	Phys-diag	Yes	45/ 264,909	16.99					
Västmanlands län, Kingdom of Sweden(72)	1934	59.79	Phys-diag	Yes	33/ 161,708	20.41					
Värmlands län, Kingdom of Sweden(72)	1934	59.87	Phys-diag	Yes	75/ 369,945	20.27					
Värmlands län, Kingdom of Sweden(84)	2002	59.87	Poser(25)	Yes	465†/ 273,427	170.07	209.97	103.17	123.43	235.97	288.67
East End, Oslo, Kingdom of Norway(85)	1995	59.93	Poser(25)	Yes	88/ 74,397	118.28					

[8] The DMSR criteria are an amalgam of the Allison & Millar and McAlpine criteria, with the definite group being equivalent to the Schumacher criteria, ignoring the age limit.
[9] Allison & Millar criteria used for cases with onset prior to 1994; Poser criteria used for cases with onset from 1994 onwards
[10] Paraclinical evidence ignored and not used in making diagnosis
[11] Including possible cases as defined by McDonald & Halliday criteria
[12] Cases retrospectively classified using Poser criteria
[13] The Gilland criteria are a modification of the WFN criteria which sought to make more effective use of CSF diagnostic evidence.

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Oslo, Kingdom of Norway(86)	1995	59.95	Poser[14](25)	Yes	582/ 483,401	120.40	133.79	80.32	88.13	156.86	176.27
Oslo, Kingdom of Norway(87)	2005	59.95	Poser[14](25)	Yes	786/ 529,846	148.34	166.65	95.59	104.82	198.56	224.19
Oslo, Kingdom of Norway: African-born(87)	2005	59.95	Poser[14](25)	Yes	4/ 20,000	20.00					
Oslo, Kingdom of Norway: Asian-born(87)	2005	59.95	Poser[14](25)	Yes	9/ 42,857	21.00					
Oslo, Kingdom of Norway: Middle east-born(87)	2005	59.95	Poser[14](25)	Yes	14/ 16,471	85.00					
Oslo, Kingdom of Norway: Norway/Western Europe-born(87)	2005	59.95	Poser[14](25)	Yes	759/ 446,471	170.00					
West End, Oslo, Kingdom of Norway(85)	1995	59.95	Poser(25)	Yes	70/ 64,186	109.06					
Uppsala län, Kingdom of Sweden(72)	1934	60.02	Phys-diag	Yes	68/ 138,060	49.25					
Uudenmaan lääni, Republic of Finland(88, 89)	1979	60.38	Schumacher(90)	Yes	337/ 625,232	53.90	58.74	41.30	47.84	65.00	68.89
Uudenmaan lääni, Republic of Finland(91)	1993	60.38	Poser(25)	Yes	1,188/ 1,277,419	93.00					
Hordaland fylke, Kingdom of Norway(92)	1960	60.41	Poser(25)	Yes	103/ 323,000	32.00					
Hordaland fylke, Kingdom of Norway(93, 94)	1963	60.41	McAlpine(82, 83)/ Schumacher(90)	Yes	70/ 394,649	20.10					
Hordaland fylke, Kingdom of Norway(94-96)	1983	60.41	McAlpine(82, 83)/ Schumacher(90)	Yes	236/ 394,649	59.80					
Hordaland fylke, Kingdom of Norway(92)	2000	60.41	Poser(25)	No	625/ 435,000	150.00					
Hordaland fylke, Kingdom of Norway(97)	2003	60.41	Poser(25)	Yes	666/ 441,660	150.79	159.79	109.80	109.45	191.30	206.63
Turku & Åland central hospital district, Republic of Finland(98, 99)	1964	60.44	Allison & Millar(6)	Yes	131†/ 425,000	30.82	34.95				
Helsinki central hospital district, Republic of Finland[97, 98]	1964	60.46	Allison & Millar(6)	Yes	155†/ 881,000	17.59	19.31				
Shetland Islands, Scotland, UK(67, 68)	1954	60.50	Sutherland(67)	Yes	25†/ 18,715	133.58					
Shetland Islands, Scotland, UK(68, 69)	1962	60.50	WFN(4)	Yes	29†/ 17,537	165.37					
Shetland Islands, Scotland, UK(68, 70)	1970	60.50	Allison & Millar(6)	Yes	31†/ 17,535	176.79					
Shetland Islands, Scotland, UK(68, 70)	1974	60.50	Allison & Millar(6)	Yes	34†/ 18,445	184.33					
Shetland Islands, Scotland, UK(70)	1977	60.50	Allison & Millar(6)	Yes	34/ 18,478	184.00					
Shetland Islands, Scotland, UK(100)	1984	60.50	Allison & Millar(6)	Yes	40†/ 23,529	170.00					
Southwest Finland region, Republic of Finland(101)	1963	60.61	Allison & Millar(6)	Yes	129†/ 395,706	32.60					
Hämeenlinna central hospital district, Republic of Finland(98, 99)	1964	60.81	Allison & Millar(6)	Yes	23†/ 136,800	16.81	19.27				
Kotka central hospital district, Republic of Finland(98, 99)	1964	60.83	Allison & Millar(6)	Yes	31†/ 193,600	16.01	19.38				
Kopparbergs län, Kingdom of Sweden(72)	1934	61.06	Phys-diag	Yes	50/ 249,647	20.03					
Lahti central hospital district, Republic of Finland(98, 99)	1964	61.21	Allison & Millar(6)	Yes	36/ 177,000	20.34	23.96				
Gävleborgs län, Kingdom of Sweden(72)	1934	61.28	Phys-diag	Yes	45/ 279,588	16.10					
South Saimaa central hospital district, Republic of Finland(98, 99)	1964	61.29	Allison & Millar(6)	Yes	24/ 134,600	17.83	21.56				
Satakunta central hospital district, Republic of Finland(98, 99)	1964	61.53	Allison & Millar(6)	Yes	50†/ 230,000	21.74	25.25				
Tampere central hospital district, Republic of Finland(98, 99)	1964	61.71	Allison & Millar(6)	Yes	89†/ 388,100	22.93	26.84				
Mikkeli central hospital district, Republic of Finland(98, 99)	1964	61.85	Allison & Millar(6)	Yes	16†/ 122,000	13.11	16.66				
Færøerne, Kingdom of Denmark(102, 103)	1950	62.00	Schumacher[15](90)	Yes	13[b]/ 31,785	40.90					

[14] MRI-based paraclinical evidence not used in making diagnosis
[15] Cases retrospectively classified using Schumacher criteria

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Færøerne, Kingdom of Denmark(102, 103)	1961	62.00	Schumacher(90)	Yes	22/ 34,591	63.60					
Færøerne, Kingdom of Denmark(104)	1960	62.00	WFN(4)	Yes	19†/ 35,122	54.10					
Færøerne, Kingdom of Denmark(102, 103)	1972	62.00	Schumacher(90)	Yes	15/ 39,164	38.30					
Færøerne, Kingdom of Denmark(102, 103)	1977	62.00	Schumacher(90)	Yes	14/ 41,543	33.70					
Færøerne, Kingdom of Denmark: Native Faroese(105)	1998	62.00	Poser(25)	Yes	32/ 44,262	72.30	84.58	39.40	44.10	107.38	122.25
Savonlinna central hospital district, Republic of Finland(98, 99)	1964	62.06	Allison & Millar(6)	Yes	12†/ 87,800	13.67	17.05				
Central Finland central hospital district, Republic of Finland(98, 99)	1964	62.58	Allison & Millar(6)	Yes	40†/ 248,500	16.10	19.90				
Central Finland lääni, Republic of Finland(106)	1983	62.58	Poser[12](25)	Yes	96/ 246,154	39.00		24.00		52.00	
Central Finland lääni, Republic of Finland(106)	1993	62.58	Poser(25)	Yes	153/ 259,322	59.00		35.00		82.00	
Central Finland lääni, Republic of Finland(106)	2000	62.58	Poser(25)	Yes	277/ 263,810	105.00		61.00		148.00	
Seinajoki central hospital district, Republic of Finland(98, 99)	1964	62.74	Allison & Millar(6)	Yes	82†/ 209,900	39.07	49.08				
Møre og Romsdal fylke, Kingdom of Norway(107)	1961	62.75	WFN(4)	Yes	81†/ 213,900	37.87	30.92	38.35	29.17	37.38	27.14
Møre og Romsdal fylke, Kingdom of Norway(108)	1961	62.75	McAlpine[10](82, 83)	Yes	69†/ 214,000	32.25					
Møre og Romsdal fylke, Kingdom of Norway(108)	1985	62.75	McAlpine[10](82, 83)	Yes	237†/ 237,237	99.88					
North Karelia lääni, Republic of Finland(98, 99)	1964	62.75	Allison & Millar(6)	Yes	24/ 203,200	11.81	14.19				
Seinajoki seutukunta, Republic of Finland(91)	1993	62.78	Poser(25)	Yes	370/ 196,809	188.00					
Vaasa central hospital district, Republic of Finland(98, 99)	1964	62.87	Allison & Millar(6)	Yes	49†/ 163,000	30.06	34.55				
Vaasan lääni, Republic of Finland(88, 89)	1979	62.90	Schumacher(90)	Yes	211/ 230,349	91.60	105.47	78.90	93.40	103.70	116.69
Vaasan lääni, Republic of Finland(91)	1993	62.90	Poser(25)	Yes	191/ 178,505	107.00					
Västernorrlands län, Kingdom of Sweden(72)	1934	62.96	Phys-diag	Yes	29/ 278,503	10.41					
Kuopio central hospital district, Republic of Finland(98, 99)	1964	63.15	Allison & Millar(6)	Yes	32/ 269,300	11.88	14.51				
Jämtlands län, Kingdom of Sweden(72)	1934	63.37	Phys-diag	Yes	25/ 134,500	18.59					
Central Ostrobothnia central hospital district, Republic of Finland(98, 99)	1964	63.69	Allison & Millar(6)	Yes	29†/ 119,400	24.29	31.30				
Nord-Trondelag fylke, Kingdom of Norway (109)	2000	64.14	Poser(25)	Yes	208/ 127,139	163.60					
Västerbottens län, Kingdom of Sweden(72)	1934	64.50	Phys-diag	Yes	63/ 204,035	30.88					
Västerbottens län, Kingdom of Sweden(91)	1990	64.50	Poser(25)	Yes	313/ 250,400	125.00	136.82	86.00	93.04	163.00	177.56
Västerbottens län, Kingdom of Sweden(110)	1997	64.50	Poser(25)	Yes	399/ 259,163	154.00	168.74	105.00	112.20	202.00	221.35
Kainuu central hospital district, Republic of Finland(98, 99)	1964	64.63	Allison & Millar(6)	Yes	12†/ 108,900	11.02	14.98				
Republic of Finland(111)	1972	64.95	Schumacher(90)	Yes	1,866/ 4,708,546	39.60	36.99	36.12		42.92	
Republic of Iceland(112)	1955	65.00	Allison & Millar(6)	Yes	69†/ 158,000	43.67	55.67	58.32	45.49	67.90	65.13
Republic of Iceland(113)	1955	65.00	Schumacher(90)	Yes	91†/ 159,480	52.83	69.92	41.42	58.24	65.69	80.79
Republic of Iceland(114)	1960	65.00	Schumacher(90)	Yes	102†/ 175,680	58.06	77.46	49.61	66.79	66.68	87.38
Republic of Iceland(114)	1965	65.00	Schumacher(90)	Yes	110†/ 193,184	56.94	78.10	48.14	68.15	65.93	87.36
Republic of Iceland(115, 116)	1989	65.00	Poser(25)	Yes	252/ 264,000	95.46					
Oulu central hospital district, Republic of Finland(98, 99)	1964	65.11	Allison & Millar(6)	Yes	53†/ 256,300	20.68	28.37				
Nordland fylke, Kingdom of Norway(117)	1999	66.75	Poser(25)	Yes	252/ 238,547	53.00	65.57	68.79	40.79	142.45	88.63
Kemi central hospital district, Republic of Finland(98, 99)	1964	67.04	Allison & Millar(6)	Yes	12†/ 88,200	13.61	17.12				
Norrbottens län,	1934	67.15	Phys-diag	Yes	22/	11.01					

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Kingdom of Sweden(72)					199,825						
Lapland central hospital district, Republic of Finland(98, 99)	1964	67.91	Allison & Millar(6)	Yes	19†/ 128,100	14.83	23.16				
Troms and Finnmark fylker, Kingdom of Norway(118)	1973	69.25	Allison & Millar(6)	Yes	44†/ 213,116	20.21	23.40	20.02	26.07	20.40	20.92
Troms and Finnmark fylker, Kingdom of Norway(119)	1983	69.25	Rose(2)	Yes	71†/ 225,073	31.55	34.53	25.21	26.91	38.16	41.61
Troms and Finnmark fylker, Kingdom of Norway(120)	1993	69.25	Poser(25)/ Rose(2)	Yes	164/ 224,724	73.01	80.76				

Appendix 3A.4 Atlantic and Central Europe

La Palma Island, Islas Canarias, Kingdom of Spain(121)	1998	28.10	Poser(25)	Yes	31/ 81,507	42.00		22.00		60.00	
Las Palmas, Islas Canarias, Kingdom of Spain(122)	1982	28.22	Schumacher(90)	Yes	44/ 700,636	6.28					
Las Palmas, Islas Canarias, Kingdom of Spain(123)	2002	28.22	Poser(25)/ McDonald#(26)	Yes	64/ 82,623	77.46	73.20	39.56	38.06	113.79	105.89
Lanzarote, Las Palmas, Islas Canarias, Kingdom of Spain(124)	1987	29.04	Numerical Poser(125)	Yes	9/ 60,000	15.00	23.83	19.79	35.29	10.11	13.15
Vélez-Málaga Sanitary District, Kingdom of Spain(126, 127)	1991	36.77	Poser(25)	Yes	19/ 36,014	52.76	63.40	32.73	41.66	73.52	83.63
Alcoy Health District, Kingdom of Spain(128, 129)	1988	38.73	Rose(2)	Yes	23/ 133,915	17.18	19.17				
Alcoy Health District, Kingdom of Spain(130, 131)	1997	38.73	Poser(25)	Yes	54/ 130,786	41.29	47.10	20.35	23.46	61.27	69.10
Marina Alta Health District, Kingdom of Spain(132)	2001	38.77	Poser(25)	Yes	54/ 129,426	41.72					
Santarém, Portuguese Republic(133, 134)	1998	39.20	Poser(25)	Yes	29/ 62,621	46.31	50.82	23.29	27.31	67.56	72.71
Menorca, Islas Baleares, Kingdom of Spain(135)	1996	40.23	Poser(25)	Yes	46/ 67,009	68.65	79.62	42.14	48.32	94.72	108.76
Móstoles, Kingdom of Spain(136)	1996	40.32	Poser(25)	Yes	85/ 195,979	43.37	40.98	32.90	30.04	53.68	51.17
Teruel, Kingdom of Spain(137)	1985	40.35	Poser(25)	Yes	12†/ 153,673	7.80					
Teruel province, Kingdom of Spain(138)	1996	40.60	Poser(25)	Yes	46/ 143,680	32.02	38.29	23.53	28.47	40.60	47.43
Bajo Aragón, Kingdom of Spain(139)	2003	40.67	Poser(25)	Yes	44/ 58,666	75.00		50.00		99.70	
Aragón, Kingdom of Spain(137)	1985	41.00	Poser(25)	Yes	115†/ 1,229,611	9.10					
Calatayud Sanitary District, Kingdom of Spain(140)	1995	41.31	Poser(25)	Yes	34/ 58,591	58.00	69.90	37.50	42.60	78.50	95.30
Costa de Ponent, Cataluña, Kingdom of Spain(141)	2001	41.38	Poser(25)	No	633/ 1,176,143	53.82		41.53		65.81	
Valladolid, Kingdom of Spain(142)	1997	41.65	Poser(25)	Yes	54/ 92,632	58.30	54.18	40.68	38.88	74.41	68.42
Zaragoza, Kingdom of Spain(137)	1985	41.66	Poser(25)	Yes	93†/ 859,406	10.80					
Barcelona, Kingdom of Spain(126)	1991	41.83	Poser(25)	No	41/ 71,985	57.00		40.00		73.00	
Osona, Kingdom of Spain(143)	1991	41.83	Poser(25)	Yes	42/ 71,985	58.35	64.23	40.01	45.58	75.69	81.59
Huesca, Kingdom of Spain(137)	1985	42.13	Poser(25)	Yes	10†/ 216,532	4.60					
Corse département, French Republic: French Farmers(144)	2003	42.15	Phys-diag	Yes	9/ 266,000	53.00		56.20		52.60	
Corse département, French Republic(145)	2004	42.15	Poser(25)	No	109†/ 209,615	52.00					
Alt Empordà, Cataluña, Kingdom of Spain(146)	2006	42.27	Phys-diag	No	78/ 124,000	62.30					
Hautes-Pyrénées département, French Republic(147)	1983	42.50	Poser(25)	Yes	63/ 157,500	40.00					
Comunidad Foral de Navarra, Kingdom of Spain(148)	1986	42.62	Poser(25)	Yes	84/ 316,344	26.55	27.43				
Gijón Health District, Asturias, Kingdom of Spain(149)	1994	43.00	Poser(25)	Yes	21/ 33,775	65.14	62.22	59.97	57.62	70.18	66.50
Gijón, Asturias, Kingdom of Spain(150)	1989	43.00	Poser(25)	Yes	19/ 81,462	23.32					
Marseille, French Republic(151, 152)	1960	43.25	Behrend(151)	Yes	93/ 661,500	14.06					

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Pyrénées-Atlantiques département, French Republic(153)	1988	43.25	Poser(25)	No	216/ 578,141	34.10		
Haute-Garonne, French Republic(154)	2005	43.31	Poser(25)/ McDonald[6](26)	Yes	1,226/ 1,155,838	106.07		
Midi- Pyrénées Région, French Republic French Farmers(144)	2003	43.50	Phys-diag	Yes	148/ 2,638,000	51.00	35.60	70.20
Languedoc -Roussillon Région, French Republic French Farmers(144)	2003	43.67	Phys-diag	Yes	125/ 2,402,000	53.20	34.80	76.40
Avignon, French Republic(155)	1984	44.00	Phys-diag	Yes	81/ 165,992	48.60	38.60	123.50
Provence-Alpes-Côte d'Azur Région, French Republic French Farmers(144)	2003	44.00	Phys-diag	Yes	112/ 4,666,000	50.90	35.90	71.10
Aquitaine Région, French Republic French Farmers(144)	2003	44.58	Phys-diag	Yes	184/ 2,988,000	55.40	41.60	130.00
Auvergne Région, French Republic French Farmers(144)	2003	45.33	Phys-diag	Yes	115/ 1,314,000	78.40	28.30	83.10
Rhône-Alpes Région, French Republic French Farmers(144)	2003	45.36	Phys-diag	Yes	193/ 5,814,000	70.50	34.70	102.40
Limousin Région, French Republic French Farmers(144)	2003	45.69	Phys-diag	Yes	74/ 711,000	76.10	38.10	109.60
Poitou-Charentes Région, French Republic French Farmers(144)	2003	46.08	Phys-diag	Yes	101/ 1,668,000	46.80	34.50	59.20
Kanton Wallis, Swiss Confederation(156)	1923	46.21	Phys-diag	Yes	4/ 128,246	3.12		
Kanton Wallis, Swiss Confederation(157, 158)	1957	46.21	Phys-diag	Yes	32/ 169,600	18.87		
République et Canton de Genève, Swiss Confederation(156)	1923	46.22	Phys-diag	Yes	35/ 171,000	20.47		
République et Canton de Genève, Swiss Confederation(157)	1957	46.22	Phys-diag	Yes	80/ 231,300	34.59		
Repubblica e Cantone Ticino, Swiss Confederation(156)	1923	46.22	Phys-diag	Yes	11/ 152,256	7.22		
Repubblica e Cantone Ticino, Swiss Confederation(156)	1957	46.22	Phys-diag	Yes	38/ 182,000	20.88		
Canton de Vaud, Swiss Confederation(156)	1923	46.54	Phys-diag	Yes	36/ 317,498	11.34		
Canton de Vaud, Swiss Confederation(157)	1957	46.54	Phys-diag	Yes	177/ 399,900	44.26		
Kanton Graubünden, Swiss Confederation(156)	1923	46.58	Phys-diag	Yes	14/ 119,854	11.68		
Kanton Graubünden, Swiss Confederation(157)	1957	46.58	Phys-diag	Yes	49/ 143,000	34.27		
Kanton Obwalden, Swiss Confederation(156)	1923	46.66	Phys-diag	Yes	2/ 17,567	11.38		
Etat de Fribourg, Swiss Confederation(156)	1923	46.71	Phys-diag	Yes	10/ 143,055	6.99		
Etat de Fribourg, Swiss Confederation(157)	1957	46.71	Phys-diag	Yes	55/ 163,500	33.64		
Kanton Unterwalden, Swiss Confederation(157)	1957	46.71	Phys-diag	Yes	11/ 43,600	25.23		
Kanton Nidwalden, Swiss Confederation(156)	1923	46.74	Phys-diag	Yes	1/ 13,956	7.17		
Kanton Uri, Swiss Confederation(157)	1957	46.76	Phys-diag	Yes	7/ 30,100	23.26		
Kanton Glarus, Swiss Confederation(156)	1923	46.97	Phys-diag	Yes	9/ 33,834	26.60		
Kanton Glarus, Swiss Confederation(157)	1957	46.97	Phys-diag	Yes	19/ 39,100	48.59		
République et Canton de Neuchâtel, Swiss Confederation(156)	1923	46.99	Phys-diag	Yes	26/ 131,349	19.79		
République et Canton de Neuchâtel, Swiss Confederation(157)	1957	46.99	Phys-diag	Yes	82/ 141,100	58.11		
Kanton Bern, Swiss Confederation(156)	1923	47.00	Phys-diag	Yes	137/ 674,394	20.31		
Kanton Bern, Swiss Confederation(157)	1957	47.00	Phys-diag	Yes	473/ 853,600	55.41		

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Kanton Bern, Swiss Confederation(159, 160)	1986	47.00	Poser(25)	Yes	1,016/ 920,289	110.40	125.93	82.00	94.03	137.30	155.61
Chalon-sur-Saône, French Republic(155)	1984	47.00	Phys-diag	Yes	90/ 156,256	58.50					
Bourgogne Région, French Republic French Farmers(144)	2003	47.00	Phys-diag	Yes	100/ 1,612,000	70.10		43.90		104.00	
Franche-Comté Région, French Republic French Farmers(144)	2003	47.00	Phys-diag	Yes	67/ 1,130,000	95.40		41.90		160.90	
Kanton Luzern, Swiss Confederation(161)	1923	47.03	Phys-diag	Yes	45/ 177,073	25.41					
Kanton Luzern, Swiss Confederation(157)	1957	47.03	Phys-diag	Yes	126/ 245,000	51.43					
Kanton Schwyz, Swiss Confederation(156)	1923	47.08	Phys-diag	Yes	5/ 59,731	8.37					
Kanton Schwyz, Swiss Confederation(157)	1957	47.08	Phys-diag	Yes	37/ 75,000	49.33					
Kanton Zug, Swiss Confederation(156)	1923	47.16	Phys-diag	Yes	4/ 31,569	12.67					
Kanton Zug, Swiss Confederation(157)	1957	47.16	Phys-diag	Yes	30/ 46,800	64.10					
Kanton St. Gallen, Swiss Confederation(156)	1923	47.20	Phys-diag	Yes	37/ 295,543	12.52					
Kanton St. Gallen, Swiss Confederation(157)	1957	47.20	Phys-diag	Yes	138/ 330,000	41.82					
Kanton Solothurn, Swiss Confederation(161)	1923	47.31	Phys-diag	Yes	23/ 130,617	17.61					
Kanton Solothurn, Swiss Confederation(157)	1957	47.31	Phys-diag	Yes	103/ 188,700	54.58					
Kanton Appenzell, Swiss Confederation(156)	1923	47.33	Phys-diag	Yes	4/ 69,968	5.72					
Kanton Appenzell, Swiss Confederation(157)	1957	47.33	Phys-diag	Yes	24/ 62,400	38.46					
Kanton Aargau, Swiss Confederation(161)	1923	47.40	Phys-diag	Yes	84/ 240,776	34.89					
Kanton Aargau, Swiss Confederation(157)	1957	47.40	Phys-diag	Yes	189/ 330,400	56.69					
Côte-d'Or département, French Republic(162)	1983	47.42	Schumacher(90)	Yes	177/ 478,378	37.00					
Kanton Zürich, Swiss Confederation(156)	1923	47.44	Phys-diag	Yes	209/ 538,602	38.80					
Kanton Zürich, Swiss Confederation(157)	1957	47.44	Phys-diag	Yes	519/ 876,200	59.23					
Kanton Basel-Landschaft, Swiss Confederation(161)	1923	47.46	Phys-diag	Yes	25/ 82,390	30.34					
Kanton Basel-Landschaft, Swiss Confederation(157)	1957	47.46	Phys-diag	Yes	87/ 124,300	69.99					
Kanton Basel-Landschaft, Swiss Confederation(163)	1985	47.46	McAlpine(82, 83)	Yes	313/ 226,000	138.50					
Pays de la Loire Région, French Republic French Farmers(144)	2003	47.47	Phys-diag	Yes	207/ 3,312,000	59.30		25.10		97.30	
Centre Région, French Republic French Farmers(144)	2003	47.50	Phys-diag	Yes	175/ 2,467,000	84.20		57.60		110.90	
Kanton Thurgau, Swiss Confederation(156)	1923	47.55	Phys-diag	Yes	26/ 135,933	19.13					
Kanton Thurgau, Swiss Confederation(157)	1957	47.55	Phys-diag	Yes	79/ 158,600	49.81					
Kanton Basel-Stadt, Swiss Confederation(161)	1923	47.56	Phys-diag	Yes	104/ 140,708	73.91					
Kanton Basel-Stadt, Swiss Confederation(157)	1957	47.56	Phys-diag	Yes	230/ 217,400	105.80					
Kanton Basel-Stadt, Swiss Confederation(163)	1980	47.56	McAlpine(82, 83)	Yes	335/ 204,000	164.22					
Kanton Schaffhausen, Swiss Confederation(156)	1923	47.72	Phys-diag	Yes	10/ 50,428	19.83					
Kanton Schaffhausen, Swiss Confederation(157)	1957	47.72	Phys-diag	Yes	46/ 62,400	73.72					
Morbihan département, French Republic(164)	1978	47.83	Phys-diag	Yes	80/ 481,927	16.60		12.20		20.80	
Santiago de Compostela, Kingdom of Spain(165)	2003	47.98	Poser(25)	Yes	71/ 90,188	78.72	74.93	64.06	61.74	91.59	87.19

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Bretagne Région, French Republic French Farmers(144)	2003	48.00	Phys-diag	Yes	213/ 2,978,000	61.50		33.90		95.60	
Ille-et-Vilaine département, French Republic(164)	1978	48.17	Phys-diag	Yes	163/ 631,782	25.80		16.60		34.30	
Finistère département, French Republic(164)	1978	48.25	Phys-diag	Yes	218/ 787,003	27.70		19.20		35.20	
Côtes-d'Armor département , French Republic(164)	1978	48.33	Phys-diag	Yes	141/ 230,075	26.50		17.40		33.90	
Alsace Région, French Republic French Farmers(144)	2003	48.50	Phys-diag	Yes	56/ 1,775,000	87.30					
Île-de-France Région, French Republic French Farmers(144)	2003	48.50	Phys-diag	Yes	98/ 111,111	88.20		49.50		123.10	
Bas-Rhin département , French Republic(166, 167)	1965	48.58	Phys-diag	Yes	313/ 764,424	40.94					
Lorraine Région, French Republic French Farmers(144)	2003	49.00	Phys-diag	Yes	2,718/ 2,319,000	97.40		77.80		118.70	
Lorraine Région, French Republic(168)	2004	49.00	Poser(25)	Yes	89/ 2,310,376	120.00		68.00		169.00	
Basse-Normandie Région, French Republic French Farmers(144)	2003	49.00	Phys-diag	Yes	123/ 1,436,000	77.00		47.20		113.90	
Champagne-Ardenne Région, French Republic French Farmers(144)	2003	49.00	Phys-diag	Yes	144/ 1,337,000	99.60		50.20		149.00	
Jihocesky kraj, Czech Socialist Republic(169, 170)	1984	49.06	Poser(25)	Yes	549/ 694,674	79.03					
Jihomoravsky kraj, Czech Socialist Republic(169, 170)	1984	49.09	Poser(25)	Yes	1,627/ 2,056,238	79.13					
Haute-Normandie Région, French Republic French Farmers(144)	2003	49.50	Phys-diag	Yes	68/ 1,787,000	82.50		54.90		99.80	
Picardie Région, French Republic French Farmers(144)	2003	49.50	Phys-diag	Yes	139/ 1,869,000	103.20		62.80		144.00	
Severomoravský kraj, Czech Socialist Republic(169, 170)	1984	49.57	Poser(25)	Yes	1,149/ 1,954,904	58.78					
Západočeský kraj, Czech Socialist Republic(169, 170)	1984	49.70	Poser(25)	Yes	569/ 874,324	65.08					
46 communities in and around Spessart, Federal Republic of Germany(171)	1958	49.88	Phys-diag	Yes	75/ 79,787	94.00					
Darmstadt, Federal Republic of Germany(172)	1982	49.98	German Poser(173-175)	Yes	328/ 611,940	53.60					
Southern Hesse, Federal Republic of Germany(176)	1980	49.98	German Poser(173-175)	Yes	324/ 612,600	52.89	54.76	38.21	39.46	66.82	68.99
Southern Hesse, Federal Republic of Germany: German-born(176)	1980	49.98	German Poser(173-175)	Yes	316/ 549,600	57.50					
Southern Hesse, Federal Republic of Germany: Mediterranean-born(176)	1980	49.98	German Poser(173-175)	Yes	8/ 63,000	12.70					
Středočeský kraj, Czech Socialist Republic(169, 170)	1984	50.06	Poser(25)	Yes	459/ 935,153	49.08					
Východočeský kraj, Czech Socialist Republic(169, 170)	1984	50.19	Poser(25)	Yes	637/ 1,245,378	51.15					
Nord-Pas-de-Calais Région, French Republic French Farmers(144)	2003	50.47	Phys-diag	Yes	127/ 4,013,000	92.80		62.70		117.20	
Praha oblast, Czech Socialist Republic(169, 170)	1984	50.55	Poser(25)	Yes	650/ 961,005	67.64					
Severočeský kraj, Czech Socialist Republic(169, 170)	1984	50.57	Poser(25)	Yes	1,296/ 1,180,973	109.74					
Okres Teplice, Czech Republic(177)	1998	50.60	Poser(25)	Yes	115/ 88,100	130.53					
Leuven, Flemish Region, Kingdom of Belgium(178)	1991	50.88	Poser(25)	Yes	220/ 250,393	87.86	93.26	73.88	78.66	101.40	106.84
Bezirk Halle, German Democratic Republic(179)	1983	51.00	Schumacher(90)	Yes	178/ 396,529	44.90					
Bochum, Federal Republic of Germany(180)	1990	51.50	Poser(25)	No	383/ 401,500	95.00					
Göttingen,	1982	51.77	German Poser(173-175)	Yes	179/ 261,696	68.40					

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Federal Republic of Germany(172)											
Southern Lower Saxony, Federal Republic of Germany(181, 182)	1969	52.02	Bauer(183)	No	124/ 245,000	51.00					
Southern Lower Saxony, Federal Republic of Germany(184)	1975	52.02	Numerical Poser(175)	Yes	161/ 256,195	76.10		58.60		92.20	
Southern Lower Saxony, Federal Republic of Germany(181, 182)	1986	52.02	Bauer(183)	No	263/ 265,657	99.00					
Southern Lower Saxony, Federal Republic of Germany(185)	1992	52.02	Not spec.	No	342/ 261,068	131.00					
Provincie Groningen, Kingdom of the Netherlands(186)	1959	53.17	Dassel(186)	Yes	265/ 471,745	56.17	66.04	57.70	70.76	54.70	61.64
Provincie Groningen, Kingdom of the Netherlands(187)	1981	53.17	Poser[12](25)	No	281/ 559,135	50.30		38.50		61.80	
Provincie Groningen, Kingdom of the Netherlands(172)	1982	53.17	German Poser(173-175)	Yes	302/ 560,000	53.90					
Provincie Groningen, Kingdom of the Netherlands(187)	1985	53.17	Poser(25)	No	285/ 560,029	50.90					
Provincie Groningen, Kingdom of the Netherlands(187)	1992	53.17	Poser(25)	No	423/ 554,604	76.30					
Freie und Hansestadt Hamburg, Federal Republic of Germany(151)	1960	53.75	Behrend(151)	Yes	985/ 1,731,100	56.90					
German Democratic Republic(188)	1983	54.10	Poser(25)	Yes	193/ 281,341	68.60					
Rostock region, German Democratic Republic(189)	1984	54.10	Poser(25)	No	224/ 327,800	68.33					
Rostock-Stadt, Rostock-Land & Bad Doberan Landkreise, German Democratic Republic(190)	177	54.12	Schumacher(90)	Yes	186/ 308,089	60.37					
Rostock-Stadt, Rostock-Land & Bad Doberan Landkreise, German Democratic Republic(191)	1983	54.12	Poser(25)	Yes	193/ 281,341	68.60		50.00		86.00	
Stralsund region, German Democratic Republic(189)	1988	54.30	Poser(25)	Yes	116/ 187,600	62.30					

Appendix 3A.5 Italian region

Città di Agrigento, Sicilia, Italian Republic(192)	1975	37.32	Phys-diag	Yes	17/ 49,979	34.00					
Comune di Caltanissetta, Sicilia, Italian Republic(193)	1981	37.45	Allison & Millar(6)	Yes	33/ 60,661	51.06	51.78	34.82	36.41	65.63	66.09
Comune di Caltanissetta, Sicilia, Italian Republic(194)	2002	37.45	Poser(25)	Yes	101/ 60,919	165.79	171.27	107.58	113.03	218.04	225.45
Comune di Catania, Sicilia, Italian Republic(195)	1989	37.50	Rose(2)	Yes	79†/ 380,328	20.77		22.32		19.32	
Comune di Catania, Sicilia, Italian Republic(196)	1995	37.50	Poser(25)	Yes	195/ 333,255	58.51	65.04	54.78	63.73	61.91	66.26
Comune di Catania, Sicilia, Italian Republic(197)	1999	37.50	Poser(25)	Yes	288/ 313,110	91.81	96.13	80.38	86.00	102.01	105.57
Comune di Enna, Sicilia, Italian Republic(198)	1975	37.56	Phys-diag	Yes	15/ 28,189	53.21	53.80	21.80	23.32	82.10	82.16
Comune di Enna, Sicilia, Italian Republic(199)	1995	37.56	Poser(25)	Yes	34/ 28,273	120.26	124.06	110.55	118.17	129.22	129.54
Comune di Linguaglossa, Sicilia, Italian Republic(200)	1991	37.85	Poser(25)	Yes	2/ 5,560	35.97					
Comune di Linguaglossa, Sicilia, Italian Republic(200)	2001	37.85	Poser(25)	Yes	11/ 5,422	202.88	214.02	154.26	167.33	247.44	257.47
Provincia di Messina, Sicilia, Italian Republic(201)	1977	38.08	Allison & Millar(6)	Yes	85/ 673,791	12.62					
Città di Bagheria, Sicilia, Italian Republic(202)	1994	38.08	Poser(25)	Yes	25/ 50,607	49.40					
Comune di Monreale, Sicilia, Italian Republic(203)	1980	38.08	Phys-diag	Yes	11/ 23,305	43.30	52.67	47.95	56.53	38.79	49.08
Comune di Monreale, Sicilia, Italian Republic(204)	1991	38.08	Poser(25)	Yes	19/ 26,256	72.36	88.37	62.14	76.31	82.20	99.59
Comune di Monreale, Sicilia, Italian Republic(205)	2000	38.08	Poser(25)	Yes	21/ 29,493	71.20	80.15	48.46	55.29	93.04	103.27
Southwest Sardegna, Italian Republic(206)	2005	39.33	Polman[3](15)	Yes	239/ 139,669	171.00		114.00		227.00	
Provincia di Cagliari, Sardegna, Italian Republic(207)	1964	39.37	McAlpine(82, 83)	Yes	90†/ 774,680	11.61					
Sardegna, Italian Republic(208)	1971	40.17	Allison & Millar(6)	Yes	184/ 1,473,800	144.40	14.45	9.03	10.78	15.88	17.87
Provincia di Nuoro, Sardegna, Italian Republic(207)	1964	40.20	McAlpine(82, 83)	Yes	71†/ 285,655	24.85					

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Provincia di Nuoro, Sardegna, Italian Republic(209)		1993	40.20	Poser(25)	Yes	394/ 273,768	143.90	155.50	91.60	101.55	195.11	205.70
Provincia di Nuoro, Sardegna, Italian Republic(210)		1994	40.20	Poser(25)	Yes	415/ 273,146	151.90	164.21	94.90	105.20	207.60	219.13
Provincia di Nuoro, Sardegna, Italian Republic(211)		1998	40.20	Poser(25)	Yes	428/ 272,992	156.78	169.34	96.24	105.95	216.08	228.33
Barbagia, Provincia di Nuoro , Sardegna, Italian Republic(212)		1975	40.32	Allison & Millar(6)/ Schumacher(90)	Yes	21/ 56,611	37.10		39.10		42.30	
Barbagia, Provincia di Nuoro , Sardegna, Italian Republic(213)		1981	40.32	Allison & Millar(6)	Yes	32/ 49,022	65.28					
Provincia di Salerno, Italian Republic(214)		1971	40.42	McAlpine(82, 83)	Yes	35/ 951,315	3.68					
Provincia di Salerno, Italian Republic(215)		2005	40.42	Polman[3](15)	Yes	97/ 136,667	70.98	72.66				
Città di Alghero, Sardegna, Italian Republic(208)		1980	40.48	Rose(2)	Yes	46†/ 77,981	58.99	65.00	38.90	45.62	78.40	83.03
Sorrento Peninsula, Campania, Italian Republic(216)		1998	40.64	Poser(25)	Yes	40/ 75,000	53.33					
Cava de' Tirreni, Campania, Italian Republic(216)		1998	40.70	Poser(25)	Yes	30/ 55,000	54.55					
Comune di Sassari, Sardegna, Italian Republic(217)		1985	40.73	Rose(2)	Yes	86/ 124,588	69.00	70.86	38.00	40.29	98.40	99.29
Campania region, Italian Republic(214)		1971	40.75	McAlpine(82, 83)	Yes	165/ 5,054,822	3.26					
Northwest Sardegna, Italian Republic(218)		1991	40.75	Poser(25)	Yes	276/ 184,362	102.60	114.49	59.20	71.33	144.20	154.64
Provincia di Sassari, Sardegna, Italian Republic(207)		1964	40.75	McAlpine(82, 83)	Yes	78/ 387,676	20.11					
Provincia di Sassari, Sardegna, Italian Republic(219-221)		1997	40.75	Poser(25)	Yes	686/ 454,904	144.40	147.65	82.50	86.47	205.10	204.58
Provincia di Napoli, Italian Republic(214)		1971	40.79	McAlpine(82, 83)	Yes	101/ 2,713,884	3.72					
Provincia di Napoli, Italian Republic(222)		1974	40.79	Phys-diag	Yes	255†/ 2,812,996	9.07		10.74		7.45	
Comune di Napoli, Italian Republic(216)		1998	40.83	Poser(25)	Yes	270/ 1,080,000	25.00					
Comune di Nusco, Italian Republic(223)		2001	40.88	Poser(25)	Yes	4/ 4,721	84.73					
Cita di Tempio Pausania, Sardegna, Italian Republic(224)		1987	40.90	Not spec.	No	36/ 35,128	102.48		68.80		118.60	
Provincia di Avellino, Italian Republic(214)		1971	40.97	McAlpine(82, 83)	Yes	8/ 427,479	1.87					
Provincia di Avellino, Italian Republic(222)		1974	40.97	Phys-diag	Yes	33†/ 433,864	7.60		7.87		7.33	
Provincia di Bari, Italian Republic(225)		1971	40.98	Borri(226)	Yes	186†/ 1,351,288	13.76		13.07		13.08	
Provincia di Caserta, Italian Republic(214)		1971	41.20	McAlpine(82, 83)	Yes	12/ 676,155	1.77					
Provincia di Benevento, Italian Republic(214)		1971	41.26	McAlpine(82, 83)	Yes	9/ 285,989	3.15					
Provincia di Frosinone, Italian Republic(227)		2007	41.65	Not spec.	No	467/ 491,527	95.01		53.27		134.92	
Provincia dell'Aquila, Italian Republic(228)		1996	42.10	Poser(25)	Yes	158/ 298,113	53.00	65.57	36.70	40.79	68.40	88.63
Provincia di Chieti, Italian Republic(229)		1990	42.12	Poser(25)	No	77/ 350,000	22.00					
Provincia di Pescara, Italian Republic(229)		1990	42.29	Poser(25)	No	76/ 353,488	21.50					
Comune dell'Aquila, Italian Republic(230)		1984	42.33	Schumacher(90)		22/ 66,340	33.16		12.38		52.88	
Provincia di Terni, Italian Republic(231)		1994	42.64	Poser(25)	No	137/ 223,050	61.86		40.79		81.60	
Umbria region, Italian Republic(232)		1968	42.97	Alter(18, 19)	Yes	83†/ 558,353	14.86		12.78		16.99	
Republic of San Marino(233)		1982	43.93	Phys-diag	Yes	14/ 21,322	52.00					
Republic of San Marino(234)		2005	43.93	Poser(25)	Yes	50/ 29,999	166.70		95.20		235.30	
Comune di Modena, Italian Republic(235)		1990	44.30	McAlpine(82, 83)	Yes	235/ 603,989	38.91	40.80	29.63	31.58	47.69	49.37
Reggio Emilia e Modena Province, Italian Republic(236)		1990	44.33	McAlpine(82, 83)	Yes	404/ 1,004,475	40.22	41.33	23.63	29.48	57.72	52.35
Comune di Genova, Italian Republic(237)		1997	44.40	Poser(25)	Yes	857/ 913,218	93.84	87.45	67.43	61.97	117.50	111.16
Provincia di Parme		1959	44.70	Alter(18, 19)	Yes	48†/	12.33		12.50		12.20	

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Italian Republic(238)					389,000						
Provincia di Parme, Italian Republic(239)	1980	44.70	Rose(2)	Yes	92†/ 398,759	23.07		16.02		29.70	
Provincia di Ferrara, Italian Republic(240)	1966	44.75	McAlpine(82, 83)	Yes	50†/ 396,343	12.62					
Provincia di Ferrara, Italian Republic(241)	1978	44.75	Allison & Millar(6)	Yes	104/ 386,898	26.90	28.10	25.60	27.05	28.10	29.09
Provincia di Ferrara, Italian Republic(242)	1981	44.75	Allison & Millar(6)/ Schumacher(90)	Yes	176/ 381,422	46.10	46.48	36.90	38.70	54.70	53.72
Provincia di Ferrara, Italian Republic(243)	1993	44.75	Poser(25)	Yes	249/ 358,808	69.40	68.17	46.00	45.10	90.80	89.65
Provincia di Ferrara, Italian Republic(244)	2004	44.75	Poser(25)	Yes	423/ 347,582	120.93	113.88	79.17	65.97	174.84	158.45
Comune di Copparo, Italian Republic(245)	1978	44.90	Allison & Millar(6)/ Schumacher(90)	Yes	14/ 45,153	31.00		32.90		30.10	
Città di Pavia, Italian Republic(246)	2000	45.07	Poser(25)	Yes	464/ 490,898	94.39	93.39	68.00	66.42	117.00	120.01
Città di Padova, Italian Republic(247)	1971	45.42	Allison & Millar(6)	Yes	122/ 760,649	16.04	20.08	11.27	13.99	20.62	25.75
Città di Padova, Italian Republic(248)	1999	45.42	Poser(25)	Yes	667/ 820,318	80.50		49.70		111.10	
Provincia di Venezia, Italian Republic(249)	1974	45.47	Allison & Millar(6)	Yes	170†/ 831,657	21.06	23.39	15.69	17.87	26.20	28.52
Provincia di Novara, Italian Republic(250)	1976	45.63	Alter(18, 19)	Yes	99/ 507,394	19.51		17.54		21.35	
Valle d'Aosta, Italian Republic(251)	1985	45.73	Poser(25)	Yes	44/ 114,325	39.00	30.57	17.63	16.65	45.08	43.53
Provincia di Varese, Italian Republic(252)	1975	45.85	Alter(18, 19)	Yes	191†/ 790,046	24.18					

Appendix 3A.6 Eastern Europe

Western Greece, Hellenic Republic(253)	2006	38.29	McDonald[6](26)	Yes	780/ 652,108	119.61	108.90	98.81	92.01	141.05	124.62
Nomos Kavalos & Island of Thassos, Hellenic Republic(254)	2008	40.60	Poser(25)/ McDonald[6](26)	No	56/ 145,054	38.60		32.10		44.90	
Northern Greece, Hellenic Republic(255)	1984	40.79	McDonald & Halliday[16](23)	Yes	729/ 2,467,173	29.55	30.19	25.55	27.50	33.47	32.69
Evros Prefecture, Hellenic Republic(256)	1999	41.08	Poser(25)	Yes	56/ 143,752	38.90	38.63				
Resen, The Former Yugoslav Republic of Macedonia(257)	1991	41.08	Poser(25)	No	2/ 26,624	7.51					
Ohrid, The Former Yugoslav Republic of Macedonia(257)	1991	41.12	Poser(25)	No	8/ 69,980	11.43					
Gevgelija, The Former Yugoslav Republic of Macedonia(257)	1991	41.13	Poser(25)	No	6/ 34,826	17.23					
Socialist People's Republic of Albania(258)	1988	41.14	Rose(2)	No	294/ 3,091,400	10.30					
Struga, The Former Yugoslav Republic of Macedonia(257)	1991	41.18	Poser(25)	No	10/ 66,864	14.96					
Demir Hisar, The Former Yugoslav Republic of Macedonia(257)	1991	41.27	Poser(25)	No	0/ 14,453	0					
Valandovo, The Former Yugoslav Republic of Macedonia(257)	1991	41.32	Poser(25)	No	1/ 12,457	8.03					
Prilep, The Former Yugoslav Republic of Macedonia(257)	1991	41.33	Poser(25)	No	15/ 106,989	14.02					
Kruševo, The Former Yugoslav Republic of Macedonia(257)	1991	41.37	Poser(25)	No	5/ 14,428	34.66					
Kavadarci, The Former Yugoslav Republic of Macedonia(257)	1991	41.43	Poser(25)	No	7/ 43,722	16.01					
Strumica, The Former Yugoslav Republic of Macedonia(257)	1991	41.43	Poser(25)	No	13/ 98,107	13.25					

[16] CSF oligoclonal bands and evoked responses used as paraclinical evidence in making diagnosis

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Negotino, The Former Yugoslav Republic of Macedonia(257)	1991	41.48	Poser(25)	No	4/ 23,064	17.34					
Makedonski Brod, The Former Yugoslav Republic of Macedonia(257)	1991	41.50	Poser(25)	No	1/ 13,177	7.59					
Debar, The Former Yugoslav Republic of Macedonia(257)	1991	41.52	Poser(25)	No	4/ 27,165	14.73					
Kičevo, The Former Yugoslav Republic of Macedonia(257)	1991	41.52	Poser(25)	No	6/ 58,807	10.20					
Radoviš, The Former Yugoslav Republic of Macedonia(257)	1991	41.63	Poser(25)	No	4/ 16,426	24.35					
Berovo, The Former Yugoslav Republic of Macedonia(257)	1991	41.71	Poser(25)	No	2/ 21,561	9.28					
Veles, The Former Yugoslav Republic of Macedonia(257)	1991	41.72	Poser(25)	No	12/ 71,169	16.86					
Štip, The Former Yugoslav Republic of Macedonia(257)	1991	41.73	Poser(25)	No	11/ 52,129	21.10					
Gostivar, The Former Yugoslav Republic of Macedonia(257)	1991	41.80	Poser(25)	No	11/ 120,235	9.15					
Sveti Nikole, The Former Yugoslav Republic of Macedonia(257)	1991	41.87	Poser(25)	No	1/ 22,945	4.36					
Vinica, The Former Yugoslav Republic of Macedonia(257)	1991	41.87	Poser(25)	No	4/ 20,549	19.47					
Kočani, The Former Yugoslav Republic of Macedonia(257)	1991	41.92	Poser(25)	No	8/ 52,354	15.28					
Delčevo, The Former Yugoslav Republic of Macedonia(257)	1991	41.97	Poser(25)	No	0/ 26,315	0					
Probištip, The Former Yugoslav Republic of Macedonia(257)	1991	42.00	Poser(25)	No	0/ 16,426	0					
Skopje, The Former Yugoslav Republic of Macedonia(257)	1991	42.00	Poser(25)	No	133/ 565,370	23.53					
Tetovo, The Former Yugoslav Republic of Macedonia(257)	1991	42.00	Poser(25)	No	16/ 191,680	8.35					
Kratovo, The Former Yugoslav Republic of Macedonia(257)	1991	42.07	Poser(25)	No	3/ 12,971	23.13					
Kumanovo, The Former Yugoslav Republic of Macedonia(257)	1991	42.08	Poser(25)	No	28/ 14,426	19.39					
Kriva Palanka, The Former Yugoslav Republic of Macedonia(257)	1991	42.12	Poser(25)	No	8/ 28,775	27.80					
Plovdiv, Republic of Bulgaria(259)	1992	42.15	Poser(25)	No	223/ 1,218,000	18.79	19.51	12.07	12.63	25.59	25.91
Samokov, Republic of Bulgaria(260, 261)	1998	42.33	Poser(25)	Yes	17/ 44,616	38.10					
Samokov, Republic of Bulgaria: Romani(260, 261)	1998	42.33	Poser(25)	Yes	2/ 10,869	18.40					
Samokov, Republic of Bulgaria: Whites(260, 261)	1998	42.33	Poser(25)	Yes	15/ 33,747	44.50					
Burgas, People's Republic of Bulgaria(262)	1979	42.50	Rose(2)	Yes	43/ 430,068	10.00					
Silven, People's Republic of Bulgaria(262)	1979	42.68	Rose(2)	Yes	42/ 233,897	17.96					
Sofia, People's Republic of Bulgaria(262)	1979	42.70	Rose(2)	Yes	315/ 1,133,733	27.78					
Sofia,	1998	42.70	Poser(25)	Yes	32/ 1,174,743	43.10					

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Republic of Bulgaria(260)												
Sofia, Republic of Bulgaria: Romani(260)	1998	42.70	Poser(25)	Yes	1/ 5,240	19.10						
Sofia, Republic of Bulgaria: Whites(260)	1998	42.70	Poser(25)	Yes	31/ 69,094	44.90						
Ravno, Bosnia & Herzegovina(263)	2006	42.88	Polman[3](15)	Yes	0/ 1,346	0.00						
Trojan, Republic of Bulgaria(264)	1995	42.88	Poser(25)	Yes	12/ 30,660	39.14		26.15		52.08		
Neum, Bosnia & Herzegovina(263)	2006	42.92	Polman[3](15)	Yes	0/ 4,695	0.00						
Svoge, Republic of Bulgaria(264)	1995	42.97	Poser(25)	Yes	9/ 22,913	39.28		26.14		52.48		
Dubrovnik-Neretva županije, Republic of Croatia(265)	1991	42.97	Poser(25)	Yes	20/ 126,329	15.83	19.14	11.44	14.66	19.96	23.30	
Dubrovnik-Neretva županije, Republic of Croatia(265)	2001	42.97	Poser(25)	Yes	33/ 122,847	26.86	29.72	16.84	19.22	36.24	39.49	
Veliko Tirnovo, People's Republic of Bulgaria(262)	1979	43.03	Rose(2)	Yes	59/ 349,530	16.88						
Stolac, Bosnia & Herzegovina(263)	2006	43.08	Polman[3](15)	Yes	4/ 13,351	29.96						
Čapljina, Bosnia & Herzegovina(263)	2006	43.11	Polman[3](15)	Yes	10/ 23,650	42.28						
Čitluk, Bosnia & Herzegovina(263)	2006	43.20	Polman[3](15)	Yes	4/ 15,935	25.10						
Ljubuški, Bosnia & Herzegovina(263)	2006	43.20	Polman[3](15)	Yes	6/ 24,102	24.89						
Pavlikeni, Republic of Bulgaria(261)	1998	43.24	Poser(25)	No	7/ 16,415	42.60						
Mostar, Bosnia & Herzegovina(263)	2006	43.33	Polman[3](15)	Yes	39/ 111,282	35.05						
Široki Brijeg, Bosnia & Herzegovina(263)	2006	43.37	Polman[3](15)	Yes	7/ 26,163	26.76						
Grude, Bosnia & Herzegovina(263)	2006	43.38	Polman[3](15)	Yes	1/ 15,673	6.38						
Mihaylovgrad, People's Republic of Bulgaria(262)	1979	43.42	Rose(2)	Yes	35/ 235,764	14.85						
Posušje, Bosnia & Herzegovina(263)	2006	43.47	Polman[3](15)	Yes	8/ 16,144	49.55						
Konjic, Bosnia & Herzegovina(263)	2006	43.65	Polman[3](15)	Yes	8/ 29,111	27.48						
Jablanica, Bosnia & Herzegovina(263)	2006	43.66	Polman[3](15)	Yes	3/ 11,892	25.23						
Zlatiborski okrug, Republic of Serbia(266)	2006	43.67	McDonald[6](26)	No	179/ 313,396	57.10						
Prozor-Rama, Bosnia & Herzegovina(263)	2006	43.82	Polman[3](15)	Yes	6/ 16,368	36.66						
Paraćin, Socialist Federal Republic of Yugoslavia(190)	1977	43.87	Schumacher(90)	Yes	13/ 64,718	20.09						
Rekovac, Socialist Federal Republic of Yugoslavia(190)	1977	43.87	Schumacher(90)	Yes	5/ 19,877	25.16						
Knić, Socialist Federal Republic of Yugoslavia(190)	1977	43.92	Schumacher(90)	Yes	5/ 20,965	23.85						
Ćuprija, Socialist Federal Republic of Yugoslavia(190)	1977	43.93	Schumacher(90)	Yes	7/ 38,841	18.02						
Svetozarevo, Socialist Federal Republic of Yugoslavia(190)	1977	43.97	Schumacher(90)	Yes	21/ 76,460	27.47						
Kragujevac, Socialist Federal Republic of Yugoslavia(190)	1977	43.98	Schumacher(90)	Yes	43/ 164,823	26.09						
Despotovac, Socialist Federal Republic of Yugoslavia(190)	1977	44.08	Schumacher(90)	Yes	1/ 35,690	2.80						
Giurgiu judet, Socialist Republic of Romania(267)	1984	44.14	Phys-diag	Yes	23/ 377,049	6.10		5.40		6.90		
Batočina, Socialist Federal	1977	44.15	Schumacher(90)	Yes	4/ 23,083	17.33						

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Republic of Yugoslavia(190)											
Dolj judet, Socialist Republic of Romania(267)	1984	44.21	Phys-diag	Yes	300/ 771,208	38.90		28.90		48.60	
Svilajnac, Socialist Federal Republic of Yugoslavia(190)	1977	44.22	Schumacher(90)	Yes	4/ 34,888	11.47					
Rača, Socialist Federal Republic of Yugoslavia(190)	1977	44.23	Schumacher(90)	Yes	3/ 6,262	47.91					
Topola, Socialist Federal Republic of Yugoslavia(190)	1977	44.25	Schumacher(90)	Yes	2/ 29,418	6.80					
Constanta judet, Socialist Republic of Romania(267)	1984	44.29	Phys-diag	Yes	27/ 692,308	3.90		2.00		5.90	
Aranđelovac, Socialist Federal Republic of Yugoslavia(190)	1977	44.30	Schumacher(90)	Yes	8/ 46,803	17.09					
North Adriatic Islands, Republic of Croatia(268)	1991	44.38	Poser(25)	Yes	20/ 45,372	44.08	46.30	18.10	19.87	68.76	70.88
North Adriatic Islands, Republic of Croatia(269)	2001	44.38	Poser(25)	Yes	26/ 46,372	56.07	57.07	21.97	20.57	88.95	91.03
Bucureşti, Romania(270, 271)	1977	44.42	Schumacher(90)	Yes	898†/ 1,934,052	46.43	50.54	39.87		52.64	
Ialomita judet, Socialist Republic of Romania(267)	1984	44.45	Phys-diag	Yes	33/ 302,752	10.90		9.30		12.60	
Belgrade, Federal Republic of Yugoslavia(272)	1995	44.82	Poser(25)	Yes	823/ 1,983,132	41.50		28.20		54.10	
Prahova judet, Socialist Republic of Romania(267)	1984	44.99	Phys-diag	Yes	183/ 859,155	21.30		19.40		23.90	
Argeş judet, Socialist Republic of Romania(273)	1977	45.01	Schumacher(90)	Yes	162/ 611,472	26.49	29.70				
Tulcea judet, Socialist Republic of Romania(267)	1984	45.02	Phys-diag	Yes	11/ 268,293	4.10		4.50		3.80	
Vilcea judet, Socialist Republic of Romania(267)	1984	45.03	Phys-diag	Yes	37/ 425,287	8.7		9.80		7.90	
Braila judet, Socialist Republic of Romania(267)	1984	45.05	Phys-diag	Yes	109/ 394,928	27.60		29.30		25.90	
Istria, Socialist Republic of Croatia(274)	1981	45.19	Schumacher(90)	Yes	46/ 188,332	24.42	28.81	15.16	17.61	33.34	39.71
Caras-Severin judet, Socialist Republic of Romania(267)	1984	45.21	Phys-diag	Yes	90/ 401,786	22.40		23.60		21.10	
Buzau judet, Socialist Republic of Romania(267)	1984	45.23	Phys-diag	Yes	60/ 521,739	11.50		8.70		14.30	
Calarasi judet, Socialist Republic of Romania(267)	1984	45.39	Phys-diag	Yes	19/ 339,286	5.60		4.50		7.60	
Northeast Istria, Republic of Croatia(275)	1991	45.39	Poser(25)	Yes	26/ 51,905	50.09	55.88	3.91	3.59	94.98	104.54
Northeast Istria, Republic of Croatia(276)	2001	45.39	Poser(25)	Yes	30/ 49,175	61.01	64.08	8.28	8.66	111.96	115.65
Gorski kotar, Republic of Croatia(277, 278)	1991	45.53	Poser(25)	Yes	36/ 30,545	117.86	123.82	86.61	91.41	148.05	154.37
Gorski kotar, Republic of Croatia(279)	2001	45.53	Poser(25)	Yes	40/ 26,120	153.14	157.14	117.06	118.34	187.89	193.24
Timis judet, Socialist Republic of Romania(267)	1984	45.59	Phys-diag	Yes	129/ 708,791	18.20		17.40		18.90	
Brasov judet, Socialist Republic of Romania(267)	1984	45.76	Phys-diag	Yes	281/ 669,048	42.00		22.90		61.90	
Vrancea judet, Socialist Republic of Romania(267)	1984	45.79	Phys-diag	Yes	107/ 386,282	27.70		24.50		30.90	
Galati judet, Socialist Republic of Romania(267)	1984	45.80	Phys-diag	Yes	102/ 625,767	16.30		14.20		18.40	
Hunedoara judet, Socialist Republic of Romania(267)	1984	45.83	Phys-diag	Yes	63/ 547,826	11.50		11.10		11.80	
Sibiu judet, Socialist Republic of Romania(267)	1984	45.84	Phys-diag	Yes	156/ 504,854	30.90		29.50		32.20	
Covasna judet, Socialist Republic of Romania(267)	1984	45.89	Phys-diag	Yes	87/ 226,563	38.40		26.30		50.40	
Alba judet, Socialist Republic of Romania(280)	1970	46.00	Alter(18, 19)	Yes	44†/ 390,200	11.50					
Alba judet, Socialist Republic of Romania(267)	1984	46.00	Phys-diag	Yes	103/ 423,868	24.30		21.60		27.10	
Baranya megye, People's Republic of Hungary(172)	1982	46.07	German Poser(173-175)	Yes	122/ 429,577	28.40					

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Cluj judet, Socialist Republic of Romania(280)	1970	46.11	Alter(18, 19)	Yes	164†/ 662,300	24.90					
Cluj judet, Socialist Republic of Romania(267)	1984	44.29	Phys-diag	Yes	246/ 738,739	33.30		34.00		32.70	
Varaždinska županija, Republic of Croatia(281)	1991	46.22	Poser(25)	Yes	45/ 187,155	24.04	25.00	15.37	16.18	32.28	33.21
Varaždinska županija, Republic of Croatia(281)	1997	46.22	Poser(25)	Yes	56/ 185,490	30.19	31.18	16.63	17.15	43.04	44.23
Szeged, Republic of Hungary(282)	1996	46.26	Poser(25)	Yes	129/ 198,682	65.00					
Arad judet, Socialist Republic of Romania(267)	1984	46.31	Phys-diag	Yes	79/ 500,000	15.80		13.10		18.20	
Bacau judet, Socialist Republic of Romania(267)	1984	46.42	Phys-diag	Yes	215/ 707,237	30.40		30.90		30.00	
Vaslui judet, Socialist Republic of Romania(267)	1984	46.51	Phys-diag	Yes	130/ 454,545	28.60		35.80		20.70	
Harghita judet, Socialist Republic of Romania(267)	1984	46.61	Phys-diag	Yes	94/ 353,383	26.60		21.80		31.40	
Mureş judet, Socialist Republic of Romania(280)	1970	46.63	Alter(18, 19)	Yes	153†/ 589,300	26.10					
Mureş judet, Socialist Republic of Romania(267)	1984	46.63	Phys-diag	Yes	104/ 615,385	16.90		13.50		20.30	
Mureş judet, Socialist Republic of Romania(283)	1986	46.63	Schumacher(90)	Yes	129/ 615,032	20.98		18.08		23.80	
Mureş judet, Romania(284)	2006	46.63	McDonald[6](26)	Yes	152/ 583,383	26.10	28.18				
Csongrád megye, Republic of Hungary(285)	1999	46.71	Poser(25)	Yes	218/ 201,442	62.00					
Cluj-Napoca, Socialist Republic of Romania(286)	1968	46.77	WFN(4)	Yes	84†/ 195,188	43.00					
Iasi judet, Socialist Republic of Romania(267)	1984	46.88	Phys-diag	Yes	334/ 778,555	42.90		48.60		37.40	
Neamt judet, Socialist Republic of Romania(267)	1984	46.93	Phys-diag	Yes	369/ 593,248	62.20		67.90		62.70	
Bihor judet, Socialist Republic of Romania(280)	1970	46.95	Alter(18, 19)	Yes	152†/ 604,600	25.30					
Bihor judet, Socialist Republic of Romania(267)	1984	46.95	Phys-diag	Yes	244/ 652,406	37.40		33.30		41.40	
Salaj judet, Socialist Republic of Romania(267)	1984	47.05	Phys-diag	Yes	37/ 268,116	13.80		18.90		8.90	
Fejér megye, Republic of Hungary(287)	1992	47.15	Poser(25)	No	246/ 312,662	78.70					
Bistriţa-Năsăud judet, Socialist Republic of Romania(280)	1970	47.17	Alter(18, 19)	Yes	59†/ 280,000	21.40					
Sălaj judet, Socialist Republic of Romania(280)	1970	47.17	Alter(18, 19)	Yes	47†/ 266,600	18.00					
Bistrita-Nasaud judet, Socialist Republic of Romania(267)	1984	47.22	Phys-diag	Yes	83/ 314,394	26.40		24.80		28.00	
Suceava judet, Socialist Republic of Romania(267)	1984	47.49	Phys-diag	Yes	299/ 671,910	44.50		40.10		48.80	
Maramureş judet, Socialist Republic of Romania(280)	1970	47.67	Alter(18, 19)	Yes	130†/ 454,900	28.60					
Maramures judet, Socialist Republic of Romania(267)	1984	47.67	Phys-diag	Yes	157/ 535,836	29.30		27.80		30.70	
Judeţul Satu Mare, Socialist Republic of Romania(280)	1970	47.71	Alter(18, 19)	Yes	46†/ 371,800	12.40					
Judeţul Satu Mare, Socialist Republic of Romania(267)	1984	47.71	Phys-diag	Yes	31/ 407,895	7.60		6.70		8.20	
Botosani judet, Socialist Republic of Romania(267)	1984	47.78	Phys-diag	Yes	81/ 457,627	17.70		19.60		15.90	
Rostov region, Russian Federation(288)	2007	48.01	Not spec.[17]	No	983/ 4,303,600	22.84					
Borsod-Abaúj-Zemplé megye, Republic of Hungary(289)	2006	48.03	McDonald[6](26)	No	480/ 705,882	68.00					
Gorski kotar-Kočevje region, Republic of Croatia(279)	1999	48.27	Poser(25)	Yes	87/ 57,274	151.90	153.37	127.60	126.50	175.70	178.36
Western Herzegovina, Bosnia and Herzegovina(290)	2003	48.27	McDonald[6](26)	Yes	81/ 300,746	26.90	26.47	23.00	23.87	30.00	28.89
Volgograd, Russian Federation(291)	2000	48.70	McDonald[6](26)	Yes	305/ 1,030,900	31.90					
Vinnytsia oblast,	1994	49.23	Phys-diag	No	582/ 1,889,610	30.80					

[17] Includes clinically-definite MS only

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Ukraine(292)									
Lublin, Republic of Poland(293)	1997	51.23	Poser(25)	Yes	204/ 356,064	57.29		37.90	74.30
Kępno powiat, People's Republic of Poland(294)	1981	51.27	Cendrowski(295)	Yes	5†/ 45,593	10.97	11.40		
Ostrzeszów powiat, People's Republic of Poland(294)	1981	51.42	Cendrowski(295)	Yes	11†/ 49,747	22.11	24.73		
Środa powiat, People's Republic of Poland(294, 296)	1981	51.44	Cendrowski(295)	Yes	19†/ 59,255	32.06	49.79		
Wolsztyn powiat, People's Republic of Poland(294, 296)	1981	51.55	Cendrowski(295)	Yes	29†/ 65,202	44.48	38.95		
Ostrów Wielkopolski powiat, People's Republic of Poland(294, 296)	1981	51.60	Cendrowski(295)	Yes	27†/ 75,158	35.92	39.92		
Rawicz powiat, People's Republic of Poland(294, 296)	1981	51.67	Cendrowski(295)	Yes	32†/ 55,912	57.23	55.73		
Krotoszyn powiat, People's Republic of Poland(294, 296)	1981	51.72	Cendrowski(295)	Yes	37†/ 76,028	48.67	54.28		
Kalisz powiat, People's Republic of Poland(294, 296)	1981	51.76	Cendrowski(295)	Yes	53†/ 101,935	51.99	55.00		
Gostyń powiat, People's Republic of Poland(294, 296)	1981	51.82	Cendrowski(295)	Yes	13†/ 64,038	21.53	21.67		
Leszno powiat, People's Republic of Poland(294, 296)	1981	51.84	Cendrowski(295)	Yes	21†/ 50,393	42.17	44.11		
Pleszew powiat, People's Republic of Poland(294, 296)	1981	51.92	Cendrowski(295)	Yes	12†/ 58,534	20.50	22.65		
Turek powiat, People's Republic of Poland(294, 296)	1981	52.00	Cendrowski(295)	Yes	20†/ 77,775	25.72	33.70		
Kościan powiat, People's Republic of Poland(294, 296)	1981	52.10	Cendrowski(295)	Yes	38†/ 84,494	44.97	50.36		
Pruszków, People's Republic of Poland(297, 298)	1960	52.17	Phys-diag	Yes	12/ 42,000	28.57			
Warsaw, People's Republic of Poland(297)	1957	52.23	Phys-diag	Yes	279/ 1,001,000	27.90			
Powiat Kolski, People's Republic of Poland(294, 296)	1981	52.24	Cendrowski(295)	Yes	29†/ 87,299	33.22	35.60		
Września powiat, People's Republic of Poland(294, 296)	1981	52.24	Cendrowski(295)	Yes	14†/ 62,676	22.34	22.15		
Śrem powiat, People's Republic of Poland(294, 296)	1981	52.31	Cendrowski(295)	Yes	21†/ 62,639	33.53	39.60		
Słupca powiat, People's Republic of Poland(294, 296)	1981	52.31	Cendrowski(295)	Yes	20†/ 41,010	48.77	36.47		
Nowy Tomyśl powiat, People's Republic of Poland(294, 296)	1981	52.34	Cendrowski(295)	Yes	40†/ 95,647	41.82	44.99		
Jarocin powiat, People's Republic of Poland(294, 296)	1981	52.40	Cendrowski(295)	Yes	37†/ 64,727	57.16	61.71		
Poznań, People's Republic of Poland(295)	1965	52.40	Cendrowski(295)	Yes	1,572†/ 2,547,736	60.50			
Poznań powiat, People's Republic of Poland(294, 296)	1981	52.45	Cendrowski(295)	Yes	305†/ 557,992	54.66	54.42		
Szamotuły powiat, People's Republic of Poland(294, 296)	1981	52.53	Cendrowski(295)	Yes	15†/ 69,331	21.64	49.40		
Gniezno powiat, People's Republic of Poland(294, 296)	1981	52.55	Cendrowski(295)	Yes	79†/ 63,502	124.41	125.01		
Międzychód powiat, People's Republic of Poland(294, 296)	1981	52.61	Cendrowski(295)	Yes	22†/ 43,894	50.12	56.31		
Oborniki powiat, People's Republic of Poland(294, 296)	1981	52.74	Cendrowski(295)	Yes	12†/ 63,456	18.91	19.33		
Amur oblast, Russian Soviet Federative Socialist Republic(299)	1985	52.80	Poser(25)	Yes	324/ 1,003,300	32.30		27.20	37.50
Amur oblast, Russian Federation(299)	2005	52.80	Poser(25)	Yes	287/ 887,600	32.30		25.10	39.40
Wągrowiec powiat, People's Republic of Poland(294, 296)	1981	52.81	Cendrowski(295)	Yes	34†/ 65,162	52.18	55.53		
Czarnków powiat, People's Republic of Poland(294, 296)	1981	52.94	Cendrowski(295)	Yes	15†/ 51,855	28.93	32.19		
Chodzież powiat, People's Republic of Poland(294, 296)	1981	52.95	Cendrowski(295)	Yes	16†/ 41,318	38.72	37.77		
Trzcianka powiat, People's Republic of Poland(294, 296)	1981	52.95	Cendrowski(295)	Yes	35†/ 28,936	120.96	54.78		
Bydgoszcz, People's Republic of Poland(297)	1954	53.12	Phys-diag	Yes	62/ 170,000	36.50			

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Szczecin region, Republic of Poland(300)	1992	53.43	Poser[10](25)	No	658/ 951,036	69.19					
Szczecin region, Republic of Poland(301)	1995	53.43	McAlpine(82, 83)	Yes	548†/ 990,525	55.32		45.74		64.54	
Republic of Bashkortostan, Russian Federation(302)	2004	53.85	Poser(25)	Yes	1,120/ 3,578,275	31.30					
Republic of Bashkortostan, Russian Federation: Bashkir(302)	2004	53.85	Poser(25)	Yes	80/ 888,889	9.00					
Republic of Bashkortostan, Russian Federation: Chuvash(302)	2004	53.85	Poser(25)	Yes	22/ 104,762	21.00					
Republic of Bashkortostan, Russian Federation: Russian(302)	2004	53.85	Poser(25)	Yes	237/ 1,185,000	20.00					
Republic of Bashkortostan, Russian Federation: Tatars(302)	2004	53.85	Poser(25)	Yes	234/ 806,897	29.00					
Kaunas, Republic of Lithuania(303)	2002	54.90	McDonald[6](26)	No	172/ 311,594	55.20		40.00		68.60	
Novosibirsk City, Russian Soviet Federative Socialist Republic(304)	1984	55.02	Poser(25)	Yes	398/ 1,364,400	29.20					
Novosibirsk City, Russian Federation(304)	1997	55.02	Poser(25)	Yes	693/ 1,406,274	49.30					
Novosibirsk City, Russian Federation(304)	2003	55.02	Poser(25)	Yes	694/ 1,425,600	54.40	44.20	23.88	27.31	76.16	59.91
Tiumen region, Russian Federation(305)	2002	57.15	McDonald[6](26)	Yes	731/ 3,263,393	22.40					
South Estonia, Estonian Soviet Socialist Republic(306, 307)	1989	58.60	Schumacher[18](90)	Yes	200/ 391,649	51.07					
South Estonia, Estonian Soviet Socialist Republic: Estonian natives(307)	1989	58.60	Schumacher[18](90)	Yes	180/ 325,323	55.30					
South Estonia, Estonian Soviet Socialist Republic: Non-Estonian immigrants(307)	1989	58.60	Schumacher[18](90)	Yes	14/ 52,558	26.60					
South Estonia, Estonian Soviet Socialist Republic: Non-Estonian natives(307)	1989	58.60	Schumacher[18](90)	Yes	6/ 13,768	43.60					

Appendix 3A.8 North America

State of Hawaii, USA(308)	1969	20.66	Alter(18, 19)	Yes	77†/ 778,300	9.89	12.90				
State of Hawaii, USA: Asian, born in Hawaii(308)	1969	20.66	Alter(18, 19)	Yes	20†/ 226,248	8.84	11.78				
State of Hawaii, USA: Caucasian, born in Hawaii(308)	1969	20.66	Alter(18, 19)	Yes	9†/ 856,113	10.51	14.12				
Key West, FL, USA(309)	1983	24.56	McDonald & Halliday(23)	No	37†/ 26,450	139.89					
Key West, FL, USA(310)	1985	24.56	Poser(25)	Yes	31/ 44,222	70.10					
Houston, TX, USA(311)	1959	29.76	Chipman(311)	Yes	66/ 938,219	7.03					
New Orleans, LA, USA(312)	1951	29.96	Westlund & Kurland(312)	Yes	59/ 685,000	8.61	8.75	7.62	8.31	9.52	9.17
New Orleans, LA, USA: Nonwhite only(312)	1951	29.96	Westlund & Kurland(312)	Yes	10/ 200,000	4.98	5.33	6.38	7.59	3.74	3.22
New Orleans, LA, USA: Whites only(312)	1951	29.96	Westlund & Kurland(312)	Yes	49/ 485,000	10.12	10.12	8.12	8.61	12.00	11.52
New Orleans, LA, USA(313)	1962	29.96	Westlund & Kurland(312)	Yes	813/ 1,101,956	9.55	11.02	6.24	7.84	12.61	13.97
New Orleans, LA, USA: Nonwhite only(313)	1962	29.96	Westlund & Kurland(312)	Yes	12/ 269,000	4.44	5.18	3.91	5.71	4.93	4.70
New Orleans, LA, USA:	1962	29.96	Westlund & Kurland(312)	Yes	71/ 599,000	11.85	13.22	7.27	8.60	16.13	17.52

[18] MRI/CT scans used as evidence for diagnosis

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Whites(313)											
El Paso County, TX, USA(314)	2003	31.76	Phys-diag	Yes	336/ 680,162	49.40	57.01	21.40		75.60	
Charleston County, SC, USA(19)	1955	32.82	Alter(18, 19)	Yes	26†/ 188,000	13.83	20.87				
19 counties around Lubbock, TX, USA(315, 316)	2000	33.56	Poser(25)	Yes	182/ 424,916	42.60	50.30	16.60		68.60	
19 counties around Lubbock, TX, USA: Blacks only(315-317)	2000	33.56	Poser(25)	Yes	6/ 27,173	22.10					
19 counties around Lubbock, TX, USA: Hispanic only(315-317)	2000	33.56	Poser(25)	Yes	16/ 142,448	11.20					
19 counties around Lubbock, TX, USA: Whites only(315-317)	2000	33.56	Poser(25)	Yes	140/ 249,882	56.00					
19 counties around Lubbock, TX, USA(314)	2003	33.56	Phys-diag	Yes	304/ 425,175	71.50	83.77	28.50		113.90	
Los Angeles County, CA, USA(318)	1970	34.30	Detels(319)	Yes	980/ 4,454,545	22.00					
Los Angeles County, CA, USA: Raised in California State(320)	1970	34.30	Detels(319)	Yes	334/ 1,518,182	22.00	26.95		15.19		37.89
Los Angeles County, CA, USA: Raised in Northern USA(318)	1970	34.30	Detels(319)	Yes	524/ 1,455,556		29.47		21.70		36.70
Los Angeles County, CA, USA: Raised in Northern USA(320)	1970	34.30	Detels(319)	Yes	646/ 781,829	36.00					
Los Angeles County, CA, USA: Raised in Southern USA(318)	1970	34.30	Detels(319)	Yes	94/ 391,667		15.13		9.05		20.79
Los Angeles County, CA, USA: Raised in Southern USA(320)	1970	34.30	Detels(319)	Yes	120/ 525,256	24.00					
Los Angeles County, CA, USA: US-born Japanese Americans(319)	1970	34.30	Detels(319)	Yes	9/ 93,750	9.60					
Los Alamos County, NM, USA(321)	1979	35.87	Rose(2)	Yes	14/ 18,494	75.70					
San Francisco, CA, USA(322)	1949	37.78	Kurland & Dodge(323)	Yes	316/ 1,063,973	29.70					
Jefferson County, MO, USA(324)	2002	38.26	Poser(25)	Yes	218/ 198,099	110.05					
Washington, DC, USA(325)	1958	38.88	Westlund & Kurland(312)	Yes	163†/ 788,100	20.70	22.46	16.50	18.86	24.41	25.25
Washington, DC, USA: Nonwhite only(325)	1958	38.88	Westlund & Kurland(312)	Yes	67†/ 404,000	16.61	18.00	15.03	16.69	18.07	19.18
Washington, DC, USA: Whites only(325)	1958	38.88	Westlund & Kurland(312)	Yes	96†/ 384,100	25.05	25.22	18.20	19.32	30.84	29.67
Independence & Sugar Creek, MO, USA(316, 317)	2000	39.08	Poser(25)	Yes	106/ 120,799	87.75	94.18	34.50		136.80	
Independence & Sugar Creek, MO, USA(326)	2001	39.08	Poser(25)	Yes	117/ 120,799	96.86	103.44				
Denver, CO, USA(323)	1949	39.74	Kurland & Dodge(323)	Yes	154/ 416,216	37.00	36.93	25.90	27.01	47.40	46.15
Denver, CO, USA: Blacks only(323)	1949	39.74	Kurland & Dodge(323)	Yes	2/ 5,235	11.00	10.89	21.70	22.92	0.00	0.00
Denver, CO, USA: Whites only(323)	1949	39.74	Kurland & Dodge(323)	Yes	152/ 410,810	38.20	38.08	26.10	27.19	49.50	48.22
Allegheny County, PA, USA(327)	1967	40.42	McAlpine(82, 83)	Yes	791†/ 1,454,044	54.40		43.60		64.40	
Allegheny County, PA, USA: Blacks only(327)	1967	40.42	McAlpine(82, 83)	Yes	48†/ 113,208	42.40		42.20		42.60	
Allegheny County, PA, USA: Whites only(327)	1967	40.42	McAlpine(82, 83)	Yes	694†/ 1,250,450	55.50		43.80		66.40	

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Weld County, CO, USA(328)	1982	40.48	Schumacher(90)	Yes	90†/ 123,438	72.91	98.71				
Larimer County, CO, USA(328)	1982	40.67	Schumacher(90)	Yes	141†/ 149,184	94.51	131.79				
Galion & Polk Township, OH, USA(329)	1987	40.73	Poser(25)	Yes	18/ 16,071	112.00					
Lorain County, OH, USA(316, 317)	2000	41.29	Poser(25)	Yes	320/ 284,664	112.41	124.15	59.40		163.40	
Mansfield, MA USA(330)	1971	42.03	Not spec.	Yes	14†/ 9,939	140.86					
Duxbury, MA, USA(331)	1958	42.04	Deacon(331)	Yes	15†/ 4,900	306.12					
Boston, MA, USA(332)	1948	42.36	Ipsen(332)	Yes	783†/ 1,535,294	51.00					
London, ON, Canada(333)	1984	43.00	Hader(334)	Yes	229/ 254,500	89.98	111.33	53.97	72.76	123.30	147.21
Ransomville, NY, USA(335)	1994	43.10	Poser(25)	No	8/ 1,401	571.00					
Mower County, MN, USA(336)	1978	43.67	Schumacher(90)	Yes	42/ 42,904	100.00	126.09	72.00		127.00	
Olmsted County, MN, USA(336)	1978	44.00	Schumacher(90)	Yes	90/ 89,158	102.00	142.93	49.00		151.00	
Olmsted County, MN, USA(337)	1985	44.00	Poser(25)	Yes	152/ 95,134	159.77	207.07	79.79	101.61	231.92	305.20
Olmsted County, MN, USA(338)	2000	44.00	Poser(25)	Yes	218/ 123,386	176.68	207.80	111.25	135.33	239.08	275.23
Rochester, MN, USA(339)	1965	44.02	WFN(4)	Yes	43/ 46,739	92.00	138.25				
Rochester, MN, USA(336)	1978	44.02	Schumacher(90)	Yes	61/ 56,594	108.00	158.24				
Rochester, MN, USA(337)	1985	44.02	Poser(25)	Yes	102/ 59,420	171.66	214.91	81.28	97.51	247.27	324.14
Kingston, ON, Canada(340)	1949	44.23	White & Wheelan(340)	Yes	17/ 29,825	57.00					
Halifax County, NS, Canada(19)	1955	44.85	Alter(18, 19)	Yes	64†/ 198,000	32.32	44.46				
Ottawa, ON, Canada(341)	1975	45.42	Phys-diag	Yes	222/ 331,343	67.00					
Province of Nova Scotia, Canada(342)	2001	45.69	Poser(25)	No	2,000/ 942,884	212.12					
Missoula County, MT, USA(343)	1957	45.85	Siedler(343)	Yes	25/ 42,373	59.00					
Middlesex County, ON, Canada(333)	1984	46.92	Hader(334)	Yes	324/ 356,044	91.00					
King-Pierce Counties, WA, USA(318)	1970	47.28	Detels(319)	Yes	309/ 447,826	69.00					
King-Pierce Counties, WA, USA: Raised in Northern USA(318)	1970	47.28	Detels(319)	Yes	268/ 370,631		57.13		32.38		80.16
King-Pierce Counties, WA, USA: Raised in Northern USA(320)	1970	47.28	Detels(319)	Yes	199/ 361,818	55.00					
King-Pierce Counties, WA, USA: Raised in Southern USA(320)	1970	47.28	Detels(319)	Yes	18/ 105,882	17.00					
King-Pierce Counties, WA, USA: Raised in Southern USA(318)	1970	47.28	Detels(319)	Yes	41/ 93,948		33.95		7.56		58.52
King-Pierce Counties, WA, USA: Raised in Washington State(320)	1970	47.28	Detels(319)	Yes	382/ 406,078	69.00	97.39		62.49		129.86
Cardston. AB, Canada(344)	1989	49.20	Poser(25)	Yes	7/ 7,955	88.00					
Crowsnest Pass Region, AB, Canada(344)	1989	49.63	Poser(25)	Yes	20/ 9,217	217.00					
Winnipeg, MB, Canada(312)	1951	49.90	Westlund & Kurland(312)	Yes	112/ 354,000	36.16	38.65	23.38	24.84	48.11	51.50
Winnipeg, MB, Canada(345)	1960	49.90	Westlund & Kurland(312)	Yes	128/ 354,000	35.39	40.51	27.09	31.52	43.46	48.89
Saskatoon, SK, Canada(334)	1977	52.22	Hader(334)	Yes	181†/ 135,074	134.00					
Saskatoon, SK, Canada(346)	1999	52.22	Hader(334)	No	529/ 213,306	248.00					
Saskatoon, SK, Canada(347)	2005	52.22	Poser(25)	Yes	558/ 196,815	298.26	334.83	180.73	172.12	407.06	486.22

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Province of Newfoundland & Labrador, Canada(348)	1985	53.50	Hader(334)	Yes	320/ 579,710	55.20	74.24					
Province of Newfoundland & Labrador, Canada(349)	2001	53.50	Poser(25)	Yes	493/ 522,246	94.40						
Barrhead County, AB, Canada(350)	1987	54.10	Numerical Poser(125)	Yes	19†/ 9,720	195.47	244.65					
Westlock County, AB, Canada(351)	1991	54.46	Numerical Poser(125)	Yes	23†/ 11,510	199.83	236.70	111.25	135.33	239.08	275.23	
Province of Manitoba, Canada(352)	2006	54.46	Phys-diag	Yes	3,192/ 1,148,401	278.00						
Province of Alberta, Canada(353)	1989	54.60	Phys-diag	Yes	5,548/ 2,560,222	216.70		173.10		260.30		
Province of Alberta, Canada(354)	1990	54.60	Phys-diag	Yes	5,053/ 2,578,216	217.60						
Province of Alberta, Canada(355)	1994	54.60	Phys-diag	Yes	6,617/ 2,694,339	245.59						
Province of Alberta, Canada: First nations people(355)	1994	54.60	Phys-diag	Yes	34/ 86,469	56.30						
Province of Alberta, Canada(355)	2002	54.60	Phys-diag	Yes	90/ 114,418	337.32						
Province of Alberta, Canada(355)	2002	54.60	Phys-diag	Yes	10,412/ 3,086,646	78.66						
Province of Alberta, Canada(354)	2004	54.60	Phys-diag	Yes	11,562/ 3,179,036	363.70						
Province of British Columbia, Canada(356)	1982	54.68	Schumacher(90)	Yes	3,340†/ 2,559,387	130.50		81.90		178.40		
Province of British Columbia, Canada: Chinese-Canadians(357)	2001	54.68	Poser(25)	No	41/ 264,516	15.50						

Appendix 3A.9 Latin America & the Caribbean

San Francisco de Quito, Republic of Ecuador(358)	2006	0.25	Poser(25)	Yes	103/ 2,036,260	5.06						
Santiago de Guayaquil, Republic of Ecuador(358)	2006	2.18	Poser(25)	Yes	50/ 2,206,213	2.27						
Santa Ana de los ríos de Cuenca, Republic of Ecuador(358)	2006	2.90	Poser(25)	Yes	5/ 666,085	0.75						
Departamento de Caldas, Republic of Colombia(359)	2000	3.86	Poser(25)	Yes	17/ 1,074,956	1.58						
Bogotá, Republic of Colombia(360)	2002	4.60	McDonald[6](26)	Yes	296/ 6,712,247	4.41	5.05	2.71	3.29	5.98	6.68	
Departamento del Risaralda, Republic of Colombia(359)	2000	5.09	Poser(25)	Yes	45/ 903,924	4.98						
Departamento de Santander, Republic of Colombia(359)	2000	6.32	Poser(25)	Yes	48/ 1,900,121	2.53						
Departamento de Antioquia, Republic of Colombia(359)	2000	7.14	Poser(25)	Yes	75/ 5,182,839	1.45						
Republic of Panama(361)	2005	8.45	Poser(25)/ McDonald[6][19](26)	Yes	169/ 3,228,186	5.24		1.60		8.94		
Lima, Republic of Perú(362)	2007	12.04	Poser(25)/ McDonald(26)	Yes	616/ 8,005,778	7.69						
Martinique, French Antilles, French Republic(363)	1998	14.67	Poser(25)	No	51/ 357,000	17.40						
Martinique, French Antilles, French Republic(364)	1999	14.67	McDonald[6](26)	Yes	72/ 243,000	20.99	22.29	13.21	9.23	44.27	34.43	
Guadeloupe, French Antilles, French Republic(364)	1999	16.25	McDonald[6](26)	Yes	29/ 340,000	13.36	8.77	3.85	2.61	22.12	14.50	
Vassouras, Rio de Janeiro Federative Republic of Brazil(365)	2002	22.30	Poser(25)	No	1/ 31,447	3.18						
Londrina, Federative Republic of Brazil(366)	2003	23.20	Phys-diag	No	61/ 442,029	13.80						
Arapongas, Federative Republic of Brazil(366)	2003	23.30	Phys-diag	No	11/ 78,571	14.00						
São Paulo, Federative Republic of Brazil(367)	1990	23.50	Poser(25)	Yes	486/ 11,381,733	4.27						
São Paulo, Federative Republic of Brazil(368)	1997	23.50	Poser(25)/ Fazekas(369)/Paty(370)	Yes	1,483/ 9,886,667	15.00						
San Pedro, United Mexican States(371)	2003	25.66	Poser(25)	No	38/ 125,978	30.00						
Oliva,	2003	32.00	Poser(25)/ McDonald[6](26)	No	11/ 12,400	88.00						

[19] Diagnoses prior to 2002 were made using Poser criteria; Diagnoses at and after 2002 were made using 2001 McDonald criteria

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Argentine Republic(372)											
Oriental Republic of Uruguay(373)	1998	34.54	Poser(25)	No	930/ 3,100,000	30.00					
Buenos Aires, Argentine Republic(374)	1996	34.63	Poser(25)	Yes	329/ 12,594,974	19.80					
Republic of Chile(375)	2004	36.42	Poser(25)	Yes	89/ 762,879	11.70					
Neuquén, Argentine Republic(376)	2002	38.95	Poser(25)	Yes	36/ 98,909	17.70					
Trelew, Argentine Republic(376)	2002	43.25	Poser(25)	Yes	12/ 43,984	13.40					
Argentine Patagonia, Argentine Republic(376)[20]	2002	45.50	Poser(25)	Yes	72/ 417,666		21.26	12.17	16.36	22.15	25.83
Río Gallegos, Argentine Republic(376)	2002	51.63	Poser(25)	Yes	17/ 79,144	21.50					
Ushuaia, Argentine Republic(376)	2002	54.80	Poser(25)	Yes	7/ 45,785	15.30					

Appendix 3A.10 Middle East and Africa

Republic of South Africa(377)	1960	28.12	Dean(377)	Yes	290/ 15,992,000	1.81					
Republic of South Africa: All non-whites(377)	1960	28.12	Dean(377)	Yes	2/ 993,000	0.07					
Republic of South Africa: All whites(377)	1960	28.12	Dean(377)	Yes	281/ 3,076,000	9.10	12.08	5.40	7.43	12.80	16.40
Republic of South Africa: White - Immigrants(377)	1960	28.12	Dean(377)	Yes	123/ 313,000	46.10	40.06	26.60	21.06	66.50	57.74
Republic of South Africa: White - South African-born(377)	1960	28.12	Dean(377)	Yes	158/ 2,763,000	5.70	7.48	3.30	4.47	8.00	10.28
KwaZulu Natal, Republic of South Africa(378)	2005	29.09	Polman[3](15)	Yes	167/ 10,014,500	1.67	1.89	0.91	1.14	2.38	2.58
KwaZulu Natal, Republic of South Africa: Blacks only(378)	2005	29.09	Polman[3](15)	Yes	12/ 5,316,060	0.22					
KwaZulu Natal, Republic of South Africa: Indians(378)	2005	29.09	Polman[3](15)	Yes	48/ 632,262	7.59					
KwaZulu Natal, Republic of South Africa: Mixed-race(378)	2005	29.09	Polman[3](15)	Yes	2/ 102,663	1.94					
KwaZulu Natal, Republic of South Africa: Whites(378)	2005	29.09	Polman[3](15)	Yes	105/ 409,554	25.60					
State of Qatar(379)	2002	25.30	Poser(25)	No	41/ 170,000	24.12					
State of Kuwait(380)	1984	29.42	Poser(25)	Yes	80/ 840,336	8.33					
State of Kuwait(381)	1988	29.42	Poser(25)	Yes	326/ 1,757,866	10.20					
State of Kuwait: Kuwaitis(381)	1988	29.42	Poser(25)	Yes	51/ 535,000	23.76	29.14	20.50	29.15	27.46	29.13
State of Kuwait: Palestinians(381)	1988	29.42	Poser(25)	Yes	72/ 302,000	9.51	13.70	7.17	9.55	11.81	17.56
State of Kuwait(382)	2000	29.42	Poser(25)/Paty(370)	Yes	305/ 1,745,092	18.54	17.43	12.50	10.94	28.72	23.92
State of Kuwait: Kuwaitis(382)	2000	29.42	Poser(25)/Paty(370)	Yes	247/ 555,431	44.47	39.52	39.29	35.65	49.35	43.39
State of Kuwait: Non-Kuwaitis(382)	2000	29.42	Poser(25)/Paty(370)	Yes	79/ 1,202,435	6.57	7.29	3.84	3.11	12.74	11.48
State of Israel: All immigrants(383)	1960	31.48	Alter(18, 19)	Yes	1,268†/ 28,693,898	14.47	17.67				
State of Israel: European-born immigrants(383)	1960	31.48	Alter(18, 19)	Yes	208†/ 664,581	31.30	23.31				
State of Israel: African-Asian born immigrants(383)	1960	31.48	Alter(18, 19)	Yes	61†/ 1,194,260	5.11	8.22				
State of Israel: Israel-born Israeli ancestry(384)	1981	31.48	McDonald & Halliday(23)/ Rose(2)/Poser(25)	Yes	44/ 459,800	32.40					
State of Israel: Israel-born Africasian ancestry(384)	1981	31.48	Rose(2)/McDonald & Halliday(23)/ Poser(25)	Yes	57/ 835,100	29.00					
State of Israel: Israel-born Euro/American ancestry(384)	1981	31.48	Rose(2)/McDonald & Halliday(23)/ Poser(25)	Yes	142/ 540,600	38.30					

[20] Crude prevalence for Argentine Patagonia not used in analysis due to overlap with subunit crude prevalences

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

State of Israel: Africasian immigrants(384)	1981	31.48	Rose(2)/McDonald & Halliday(23)/ Poser(25)	Yes	150/ 633,100	14.20					
State of Israel(385)	2000	31.48	Poser(25)	Yes	2,861/ 6,498,400	44.03	57.49				
State of Israel: All jews(385)	2000	31.48	Poser(25)	Yes	2,697/ 5,122,800	52.65	64.20				
State of Israel: Israel-born Jews (385)	2000	31.48	Poser(25)	Yes	1,497/ 3,165,200	47.30	85.49				
State of Israel: Europe/American-born Jews(385)	2000	31.48	Poser(25)	Yes	952/ 1,402,000	67.90	67.79				
State of Israel: Africa/Asia-born Jews(385)	2000	31.48	Poser(25)	Yes	248/ 555,600	44.64	36.00				
State of Israel: All Arabs(385)	2000	31.48	Poser(25)	Yes	164/ 846,800	11.92	20.67				
State of Israel: Moslem Arabs(385)	2000	31.48	Poser(25)	Yes	106/ 952,200	11.13					
State of Israel: Bedouins(385)	2000	31.48	Poser(25)	Yes	6/ 111,100	5.40					
State of Israel: Christian Arabs(385)	2000	31.48	Poser(25)	Yes	43/ 210,000	20.48					
State of Israel: Druze(385)	2000	31.48	Poser(25)	Yes	9/ 102,300	8.80					
Amman, Hashemite Kingdom of Jordan(386)	2004	31.58	McDonald[6](26)	Yes	156/ 402,960	39.21	35.21	22.04	21.31	56.84	48.14
Jerusalem, State of Israel: Israel-born Israel ancestry(384)	1983	31.78	Rose(2)/McDonald & Halliday(23)	Yes	21†/ 73,364	61.30					
Jerusalem, State of Israel: Israel-born Euro/American ancestry(384)	1983	31.78	Rose(2)/McDonald & Halliday(23)	Yes	23†/ 43,606	68.50					
Jerusalem, State of Israel: Israel-born Africasian ancestry(384)	1983	31.78	Rose(2)/McDonald & Halliday(23)	Yes	17†/ 75,167	51.30					
Jerusalem, State of Israel: Africasian immigrants(384)	1983	31.78	Rose(2)/McDonald & Halliday(23)	Yes	79†/ 202,872	28.60					
Jerusalem, State of Israel(387)	1995	31.78	Poser(25)	Yes	272/ 677,541	40.15					
Jerusalem, State of Israel: Israel-born Euro/American Jews(387)	1995	31.78	Poser(25)	Yes	88/ 114,966	76.54					
Jerusalem, State of Israel: Israel-born Africasian jews(387)	1995	31.78	Poser(25)	Yes	21/ 54,945	38.22					
Jerusalem, State of Israel: Europe/American-born Jews(387)	1995	31.78	Poser(25)	Yes	65/ 137,458	47.29					
Jerusalem, State of Israel: Africasian-born Jews(387)	1995	31.78	Poser(25)	Yes	79/ 202,872	38.94					
Jerusalem, State of Israel: Arabs(387)	1995	31.78	Poser(25)	Yes	19/ 167,300	11.36					
Benghazi, Great Socialist People's Libyan Arab Jamahiriya(388)	1984	32.12	McDonald & Halliday(23) McDonald[6](26)	Yes	21/ 518,745	4.05	5.87	2.99	5.36	5.18	6.34
Irbid, Hashemite Kingdom of Jordan(386)	2005	32.55		Yes	224/ 589,474	38.00					
Esfahan, Islamic Republic of Iran(389)	2005	32.63	McDonald[6](26)	Yes	1,391/ 3,923,255	35.46	40.59	15.30	18.46	56.71	61.19
Esfahan, Islamic Republic of Iran(390, 391)	2006	32.63	McDonald[6](26)	Yes	1,718/ 3,923,255	43.79	50.25	19.27	22.81	69.64	75.79
Esfahan, Islamic Republic of Iran: Armenians(390)	2006	32.63	McDonald[6](26)	Yes	15/ 8,333	180.00					
Esfahan, Islamic Republic of Iran: Persians(390)	2006	32.63	McDonald[6](26)	Yes	1,703/ 3,888,128	43.80					
Republic of Iraq(392)	2008	33.19	McDonald[6](26)	No	1,207/ 27,499,638	2.18	0.31	1.20	0.18	3.20	0.43
Republic of Malta(393)	1978	35.94	Phys-diag	Yes	17/ 322,600	5.27					
Republic of Malta(394)	1988	35.94	Poser(25)	No	29/ 345,448	8.39					

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Republic of Malta(395)	1999	35.94	Poser(25)	Yes	50/ 378,400	13.21	13.90	11.18	11.58	15.22	16.07
Maltepe, Istanbul, Republic of Turkey(396)	2005	40.95	Poser(25)/Barkhof(397)	Yes	33/ 32,531	101.40					
Edirne city, Republic of Turkey(398)	2003	41.67	Phys-diag	No	34/ 114,865	29.60					
Edirne city, Republic of Turkey Turkish-Jewish population(399)	2003	41.67	McDonald[6](26)	No	20/ 18,000	111.00					

Appendix 3A.11 Asia and Pacific Islands

Republic of the Fiji Islands(3)	1960	17.50	Phys-diag	Yes	5/ 357,143	1.40					
Bombay, Republic of India: Zorastrian population(400)	1985	18.98	Schumacher(90)	Yes	3/ 14,010	21.41	24.60				
Bombay, Republic of India: Zorastrian population(401)	1988	18.98	Schumacher(90)	Yes	14/ 50,053	27.97	28.88				
Pune, Republic of India: Zorastrian population(401)	1988	18.98	Schumacher(90)	Yes	2/ 3,448	58.00					
Hong Kong, People's Republic of China: Chinese(402)	1997	22.05	Phys-diag	Yes	53/ 6,883,117	0.77					
Republic of China(403)	2005	23.61	Phys-diag	Yes	674/ 22,770,383	2.96	3.04	1.32	1.35	4.65	4.61
Lancang Lahuzu, Yunnan Province People's Republic of China(404, 405)	1986	24.50	Phys-diag	Yes	1/ 47,825	2.10					
Naha, Okinawa Prefecture Japan(406)	1977	26.00	Japan MSRC(407)	Yes	6/ 310,000	1.90					
Okinawa Prefecture Japan(408)	1978	26.20	Japan MSRC(407)	Yes	25†/ 1,073,000	2.33					
Niigata, Japan(409, 410)	1960	28.00	Alter(18, 19)	Yes	9†/ 230,000	3.90					
Shanghai, People's Republic of China(411)	2004	31.20	McDonald[6](26)	Yes	123/ 8,848,921	1.39	1.32	0.98	0.92	1.80	1.68
Kagoshima, Japan(406)	1978	32.00	Japan MSRC(407)	Yes	4/ 417,000	0.90					
Miyazaki, Japan(406)	1978	32.00	Japan MSRC(407)	Yes	2/ 251,000	0.80					
Kumamoto City, Japan(412)	1982	32.78	Japan MSRC(407)	Yes	7/ 540,000	1.30					
Kumamoto, Japan(413)	1957	32.78	Alter(18, 19)	Yes	7†/ 332,000	2.11					
Kumamoto, Japan(409, 410)	1958	32.78	Alter(18, 19)	Yes	6†/ 332,000	1.80					
Fukuoka, Japan(409, 410)	1959	33.00	Alter(18, 19)	Yes	10†/ 608,000	1.60					
Yonago, Japan(406)	1978	35.00	Japan MSRC(407)	Yes	3/ 124,000	2.40					
Kanazawa, Japan(406)	1978	37.00	Japan MSRC(407)	Yes	6/ 401,000	1.50					
Sendai, Japan(406)	1976	38.00	Japan MSRC(407)	Yes	11/ 576,000	1.90					
Morioka, Japan(406)	1972	40.00	Japan MSRC(407)	Yes	9/ 227,000	4.00					
Hirosaki City, Japan(414)	1973	40.60	Phys-diag	Yes	6/ 159,000	3.80					
Goshogawara City, Japan(414)	1974	40.80	Phys-diag	Yes	2/ 52,000	3.90					
Aomori City, Japan(414)	1973	40.82	Phys-diag	Yes	9/ 248,000	3.60					
Aomori, Japan(406)	1978	41.00	Japan MSRC(407)	Yes	10/ 276,000	3.60					
Hirosaki, Japan(406)	1978	41.00	Japan MSRC(407)	Yes	6/ 171,000	3.50					
Sapporo, Japan(413)	1957	43.10	Alter(18, 19)	Yes	5†/ 426,000	1.17					
Sapporo, Japan(409, 410)	1958	43.10	Alter(18, 19)	Yes	7†/ 426,000	1.60					
Tokachi Province, Japan(415)	2001	43.40	Poser(25)	Yes	31/ 361,727	8.57					
Tokachi Province,	2006	43.40	Poser(25)	Yes	44/ 358,439	13.11	13.87	6.96	7.73	18.82	19.59

Appendix 3A. Supplementary Table 1. Study-specific information for all studies included in meta-analysis, organised by global region.

Japan(416)											
Asahikawa, Japan(406)	1975	44.00	Japan MSRC(407)	Yes	8/ 323,000	2.50					
Asahikawa, Japan(417)	2002	44.00	Poser(25)	Yes	37/ 363,526	10.18	10.27	7.54	8.02	12.55	12.36

Note: All place names are those used at the time of the study in question – some may no longer exist or may have been renamed.

Abbreviations:
In Australasia region: NSW=State of New South Wales; TAS=State of Tasmania; WA=State of Western Australia.
In UK region/Scandinavia and North Atlantic region: UK=United Kingdom of Great Britain & Northern Ireland.
In North America region/Asia region: AB=Province of Alberta; CA=State of California; CO= Colorado; DC=District of Columbia; FL=State of Florida; LA=State of Louisiana; MA=Commonwealth of Massachusetts; MB=Province of Manitoba; MN=State of Minnesota; MO=State of Missouri; MT=State of Montana; NM=State of New Mexico; NS=Province of Nova Scotia; NY=State of New York; OH=State of Ohio; ON=Province of Ontario; PA=Commonwealth of Pennsylvania; SC=State of South Carolina; SK=Province of Saskatchewan; TX=State of Texas; USA=United States of America; WA=State of Washington.
Diagnostic criteria: WFN=World Federation of Neurology; DMSR=Denmark Multiple Sclerosis Registry; Japan MSRC=Japan Multiple Sclerosis Research Committee

Appendix 3A.12 References

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Appendix 3B. Outline of diagnostic criteria utilised by studies included in meta-analysis, organised in approximate temporal order of publication.

Appendix 3B.1 Early criteria (pre-Allison & Millar)

Appendix 3B.1.1 Ipsen criteria: 1939-48, Boston, MA USA(1)

- **Probable MS**
    - Those cases with records presenting convincing evidence
- **Possible MS**
    - Those cases whose evidence was more doubtful.

Ipsen acknowledges these allocations are fairly arbitrary but also notes that absolutely certainty of diagnosis is impossible except by autopsy, a limitation we are similarly affected by even today.

Appendix 3B.1.2 Westlund & Kurland: 1951, Winnipeg, MT Canada & New Orleans, LA USA(2)

Diagnostic groupings as follows, with no explicit requirements for each, rather deferring to the discretion of the examining neurologist:

- Certain MS
- Probable MS
- Possible MS
    - In this class, the changes for and against MS were considered approximately even
- Doubtful, unlikely or definitely-not MS(2)

Appendix 3B.1.3 Sutherland criteria: 1954, Northern Scotland(3)

- **Probable MS**
    - This category for patients in whom the history, the results of clinical examination and, where available, hospital investigations, indicated that the diagnosis of MS was beyond reasonable doubt.
- **Possible MS**
    - Patients in whom a diagnosis of MS appears justifiable but in which the diagnosis could not be established beyond reasonable doubt;
    - This group also included patients who did not wish to be examined or could not be seen – review of hospital records for these patients suggested a diagnosis of MS
- **Rejected cases**
    - Patients found to be suffering from a disease other than MS

Appendix 3B.2 Allison & Millar Criteria and variants

Appendix 3B.2.1 Allison & Millar criteria: 1954, Northern Ireland(4)

- **Early disseminated sclerosis:**

1) this category for patients with little in the way of symptomatic presentation but with a recent history consistent with disease onset, i.e. optic neuritis, ophthalmoplegia (double vision), vertigo, sensory problems like pins & needles or numbness, or motor problems like weakness.

- **Probable disseminated sclerosis**

1) this category for patients "in which there was no reasonable doubt about the diagnosis", "usually a remitting quality", with patients presenting with physical disability "explicable only on the basis of multiple lesions"(4).

- **Possible disseminated sclerosis**

1) this category for patients in which the diagnosis was suggested by the findings but without evidence of multiple lesions in the CNS.

- **Discarded cases**

1) This category for patients in which the results of clinical examination suggested some other disease.

Appendix 3B.2.2 Siedler Criteria: 1957, Missoula County, MT USA(5)

- **Probable MS**

1) Patients whose objective documented neurologic findings were explainable only by the assumption of multiple lesions in the CNS. Historic evidence, laboratory findings and examination results could be used as supporting evidence where they supported the impression of MS and were against other diagnoses. Patients had neurologic signs and symptoms characterized by

    - exacerbations and remissions

      or

    - slow progression of lesions

- **Possible MS**

1) Patients who had insufficient evidence of multiple lesions in the CNS on the basis of neurologic examination

- **Not MS**

1) Patients in whom another diagnosis was more likely(5)

Appendix 3B.2.3 Deacon Criteria: 1958, Duxbury, MA USA(6)

- **Probable MS w/ disability**

1) This include patients with neurological signs and symptoms characterized by exacerbations and remissions or by slow progression. Patients' neurological exams were only explainable

by multiple lesions in the CNS, and patients' histories, and laboratory and neurological findings indicated MS over another diagnosis.

- **Probable MS w/o disability**

1) In this patients, clinical records revealed signs and symptoms compatible with MS which had been present in the past, but at exam there was no clear evidence of multiple lesions in the CNS

- **Possible MS**

1) This included patients in whom a diagnosis had not been entirely established.

- **Not MS**

1) This included patients in whom another diagnosis was made.

Appendix 3B.2.4 Dean Criteria: 1960, South Africa(7)

- **Probable MS**

1) Patients with a multiplicity of lesions in the CNS (DIS), usually exacerbations and remissions, and the necessary investigations to exclude other pathology had been carried out.

- **Possible MS**

1) Those patients in whom there was any doubt about the diagnosis

- **Not MS**

1) Those whose condition could be otherwise explained as non-MS(7)

Appendix 3B.3 World Federation of Neurology criteria and variants

Appendix 3B.3.1 Allison/World Federation of Neurology Criteria: 1960(8)

- **Latent or Early Probable MS**

1) Patients whose histories left little doubt as to the diagnosis but in whom there was as yet little or no disability and few neurological signs.

- **Probable MS**

1) Cases in which there was no reasonable clinical doubt as to the diagnosis; patients had to have evidence of physical disablement, usually a remitting character to their history and on examination definite physical signs explicable only on the basis of multiplicity of lesions within the neuraxis.

- **Possible MS**

1) Cases had to have some physical disablement and definite physical signs indicative of white matter disease and clinically suggestive of MS, but cases had later age of onset relative to those in probable MS group and histories were progressive or static, rather than remitting and their physical signs did not indicate such a sufficiency of lesions at different levels as was found in probable cases.

- **Discarded cases**

1) Cases in which there were no physical signs and in which documentary proof of former symptoms having occurred were deemed inadequate, or cases in which symptoms were better explained by another condition.

Appendix 3B.3.2 Alter Criteria: 1960, Halifax County, NS Canada & Charleston County, SC USA(9, 10)

- **Early Probable and Latent MS**

1) Patients showed slight or no disability and few physical signs but who had histories (presented) of remitting symptoms and signs commonly associated with the disease; patients must exhibit at least one physical sign typical of MS; patients unavailable for exam must present documented proof that signs and symptoms had occurred; optic neuritis alone was not considered MS

- **Probable MS**

1) Patients in whom there was no reasonable doubt as to the diagnosis and in whom some physical disability was found; history was usually that of remitting disease; on examination, definite physical signs that could be explained only by multiple lesions of the neuraxis were found; supporting (paraclinical) evidence such as change in colloidal gold curve (characteristic CSF) or a negative myelogram were required for acceptance of patients whose history were unreliable.

- **Possible MS**

1) Patients showing physical disability and definite physical signs indicative of CNS disease and suggestive of MS; these patients usually had progressive rather than remitting course and did not have sufficient evidence of multiple lesions at varying levels; however no other cause for the condition could be established.

Appendix 3B.3.3 Gilland criteria: 1965(11)

In 1965, Gilland proposed a modification of the WFN diagnostic criteria which would make better use of CSF-based evidence, which he felt were underrepresented in the Allison/WFN criteria. These criteria proposed a hybrid between the three groups in the WFN criteria (early probable, probable and possible) and three groupings based upon CSF findings (typical (MS characteristic), gamma-normal (found in 10-15% MS cases), and atypical (indicative of non-MS, likely infection) CSF)(11). These hybrid groupings would be thus allocated into two main groups:

- **MS Diagnostic:**

1) Probable – CSF Typical
2) Probable – CSF gamma normal
3) Probable – CSF Atypical
4) Early Probable – CSF Typical
5) Early Probable – CSF gamma normal

6) Early Possible – CSF Typical

- **MS Observation:**

1) Early Probable – CSF Atypical

2) Clinical Possible – CSF gamma normal

3) Clinical Possible – CSF Atypical

Appendix 3B.4 Poskanzer criteria and variants

Appendix 3B.4.1 Poskanzer Criteria: 1963, Northeast UK(12)

- **Probable MS**

1) Patients were included in this category when they unequivocally satisfied the DIT/DIS criteria and when there was little reasonable doubt about the diagnosis

- **Latent MS**

1) Patients in which there was little doubt about the diagnosis; however patients were asymptomatic at the time of examination, but gave a characteristic history of episodic multifocal neurological disease which defied alternative explanation

- **Possible MS**

1) Patients in which alternative diagnoses had been excluded as far as practicable and where the clinical picture was more suggestive of multiple sclerosis than other known neurological disorders(12).

Appendix 3B.4.2 Cendrowski Criteria : 1965, Western Poland(13)

- **Probable MS**

1) Patients unequivocally satisfied clinical criteria of the progressive disease with dissemination of lesions in time and space, leaving only little doubt about the diagnosis.

- **Possible MS**

1) For those patients where alternative diagnoses had been excluded as far as practicable and when the clinical picture was more suggestive for MS than of other neurological disease(13).

Appendix 3B.4.3 Hornabrook Criteria: 1971, Wellington, New Zealand(14)

- **Probable MS**

    a. Patients presenting with neurological symptoms suggestive of MS, with at least one relapse at the same site or elsewhere (DIT); neurological exam provided evidence of the presence of more than one physical lesions within the CNS, these lesions were anatomically separate (DIS)

    or

    b. Patients in whom a progressive single lesion had been succeeded by a history of relapses and scattered lesions (DIT/DIS), provided examination demonstrated the presence of physical signs consistent with various sites of damage within the CNS (DIS).

- **Latent MS**

    c. Patients presenting with a history of variable duration in which there were indications of scattered lesions in the CNS (DIS), the presence of structural damage confirmed by neurological exam, but in whom there were only mild fluctuating signs of the development of new symptoms and signs without distinctly recognizable relapses.

- **Possible MS**

    d. Patients with many of the neurological symptoms or history suggestive of MS but in whom some slightly atypical feature, such as late age of onset, made them uncertain to be MS(14).

Appendix 3B.5 Other intervening criteria

Appendix 3B.5.1 Chipman Criteria: 1959, Houston, TX USA(15)

In his 1959 study of MS in Houston, TX, Chipman made use of a standardised set of criteria for use by the various physicians providing subjects for the study, these criteria effectively being for definite/probable MS and required:

- History of remissions/relapse or temporary exacerbations of neurological symptoms
- Evidence of multiple symptomatology which could only be explained on the basis of multiple anatomical nervous system lesions
- In cases where no history or physical findings were available, the diagnosis of the examining neurologist or internist was accepted.
- Patients who were listed by the MS Society as MS cases but who had no available professional diagnosis and who refused to give a history were excluded from the study.

Appendix 3B.5.2 Behrend criteria: 1960, Marseille, France & Hamburg, Germany(16)

Cases were classified by a combination of elements including:

1) Localization of the clinically detectable foci;
    - Cerebral
    - Spinal
    - Cerebrospinal
2) Number of the clinically detectable foci;
    - One
    - Two or more
    - Uncertain
3) Course of the disease;
    - One remission

- Two or more remissions
- Chronic progressive
- Mixed

4) CSF changes

- Typical
- Atypical
- Normal
- Unknown

5) Presence of optic nerve lesion

Into groups of

- **Clinically unequivocal**
- **Probable**
- **Possible**

Appendix 3B.5.3 Dassel Criteria: 1960, Groningen Province, Netherlands(17)

The criteria for definite MS used in the study are as follows:

- Symptoms must indicate multiple lesions of the CNS
- Clinical remissions
- Changes in the CSF, i.e. a positive colloidal gold reason, normal or slightly raised protein content, increase number of cells, negative syphilitic serological reaction
- The age of symptom onset must be under 40years; if over 40, the diagnosis must be regarded with suspicion
- Subacute combined degeneration of the spinal cord must be eliminated
- No changes in the spinal column which might cause lesions of the spinal cord(17).

Appendix 3B.5.4 McAlpine Criteria: 1961, Middlesex Hospital, UK(18)

- **Definite MS**

1) A history of an acute retrobulbar neuritis or of an episode of paraesthesiae, motor weakness, double vision, unsteadiness in walking or other symptoms typical of MS which tended to improve, followed by one or more relapses during the course of years with, in addition, the presence of pyramidal and other signs indicative of

   multiple lesions in the CNS, when the patient was first seen or subsequently.

   or

2) A gradual onset of a paraplegia later followed by relapse and signs indicative of disease in brainstem, cerebrum, or optic nerve.

- **Probable MS**

1) During the original attack, clinical evidence of multiple lesions which, at the time, suggested the probability or possibility of MS, followed by good recovery. During the follow-up interval, relative or complete absence of fresh symptoms after the first year but with a tendency to variability in pyramidal and other signs originally present or the occasional late appearance of an extensor plantar response, nystagmus, tremor or temporal pallor of a disc.
2) A history of one or more attacks of acute retrobulbar neuritis accompanied or followed by pyramidal signs, usually mild in degree. Subsequently no clinical evidence of relapse.

- **Possible MS**

1) A history similar to that described under Probable (1) but with unusual features or a paucity of signs, or lack of follow-up information.
2) A history of a progressive paraplegia usually in early middle age without evidence of relapse or remission or of a lesion outside the spinal cord, appropriate investigation, including myelography, having excluded other causes of progressive paraplegia(18-20).

Appendix 3B.5.5 Schumacher Criteria: 1965(21)

6 criteria required for a diagnosis of clinically-definite MS:

- Objective abnormalities on neurological examination attributable to dysfunction of the CNS; symptoms alone are not sufficient for a diagnosis.
- At neurological exam or in medical history, there must be evidence of involvement in 2 or more separate parts of the CNS
- Objective evidence of CNS disease must be predominantly of the white matter, with more than minor gray matter involvement disqualifying.
- Involvement of the neuraxis must have occurred temporally in one of the following patterns:
    - 2 or more episode of worsening [relapse], separate by a period of one month or more, each episode lasting at least 24hrs.
    - Slow or step-wise progression of signs and symptoms over at least 6-months.
- The age of the patient must be within 10-50yrs.
- The signs and symptoms cannot be explained better by another disease process.

Appendix 3B.5.6 Danish MS Registry Criteria: 1948-64, Denmark(22)

- **Clinically-definite MS**

1) Patients satisfying the requirements for probable MS but in whom the diagnosis is maintained through the presence of additional neurological findings or symptoms.

- **Probable MS**

1) Patients with clinical signs of involvement of the CNS, which cannot be explained from a single lesion wherever it might be situated. Unequivocal physical signs of at least one lesion must be present but may for other lesions be substituted by abnormal evoked potentials or

reliable information of symptoms or physical signs in the past, adequate to localize a lesion typical of MS at a different location (DIS).

and

- The patient must show some physical disablement, a remitting quality of the history

or

- A stepwise or steady progression over at least 6-months.

- **Latent Probable MS**

1) Patients satisfying nearly all the requirements for probable MS except show slight or no disability.

and

2) A clinical history of at least 2 episodes of remitting symptoms separated by a period of at least one month (DIT)

and

- Unequivocal neurological findings, confirmed by hospital/specialist records, must fulfill the conditions of being explicable only on the basis of multiple lesions (DIS).

or

- If no subjective or objective physical signs were present at the time of the last examination, the evidence of a lesion and the documentation must be so strong that it cannot be ignored. Abnormal evoked potentials are acceptable as neurological findings, but they do not indicate self-contained lesions, if they are associated with physical signs from the same region of the white matter.

- **Possible MS**

1) Patients in whom the clinical signs of lesion of the white matter fail to prove involvement at different levels of the neuraxis (DIS), or the documentation of such involvement is insufficient.

or

2) If the course has been steady progressive from the start and symptoms and physical signs are confined to the spinal cord

and

- The progression has lasted for at least 6 months

and

- Oligoclonal bands or increased IgG –index have been detected in the CSF.

- **Discarded cases**

1) Cases in which the symptoms or findings may as well be caused by other neurological disease or cases in which the physical signs of CNS-involvement are equivocal and patients in which the suspicion of MS is unwarranted(22).

Appendix 3B.5.7 Detels Criteria: 1970, Los Angeles County, CA & King-Pierce County, WA USA(23)

- **Definite & Probable MS**

1) Symptoms referable to at least 2 areas of CNS located above and below the foramen magnum

and

2) Physician diagnosis of MS, disseminated sclerosis or demyelinating disease

and

3) Signs referable to at least 2 areas of the CNS (DIS)

and

4) Onset of symptoms at 2 separate times (DIT)

or

5) Distinct remissions and exacerbations (DIT)

- **Possible MS**

1) Symptoms referable to at least 2 areas of the CNS located above and below the foramen magnum

and

2) Diagnosis of MS by physicians

and

3) Presence of 2 signs not indicated

and

4) Onset of symptoms at 2 separate times or distinct remissions and exacerbations

- **Insufficient information**

1) Deceased, unable to complete long or short questionnaire

or

2) No physician report

- **Not MS**

1) Participants whose symptoms are better defined by another non-MS neurological condition as determined by study neurologist.(23)

Appendix 3B.5.8 Japanese MS Research Committee Criteria: 1972

The criteria described in the prevalence study by Shibasaki et al in their 1975 prevalence study of MS in Hawaii were developed by the MS Research Committee of Japan in 1972. These criteria describe MS as a condition in which:

- Probable MS

1) The age of onset is between 15-50 years
2) Symptoms and signs due to multifocal lesions in the CNS (more than two lesions in the CNS, e.g. cerebrum, spinal cord, optic nerve, etc. (DIS)
3) Remissions and exacerbations (DIT)
4) Other diseases can be excluded

- Possible MS

1) When not all the criteria required for Probable MS are met

and/or

1) In patients presenting with:

- Optic neuritis combined with other neurological symptoms such as abnormal deep reflexes, paralysis, numbness ataxia
- Myelopathy associated with ophthalmoplegia or nystagmus
- Cerebellar symptoms, spinal cord symptoms and cerebral symptoms occur successively
- Recurrent myelitis
- Recurrent optic neuritis

Appendix 3B.5.9 Bauer committee criteria: 1972(24)

In 1972, Bauer and others came together to discuss possible improvements on the Schumacher criteria, including expansion to a continuous diagnostic scale and the use of CSF-based paraclinical evidence, as well as other matters pertaining to MS diagnosis. This yielded the following generally agreed criteria:

The Schumacher criteria used in the diagnostic criteria were modified to ignore the upper age of onset limit and CSF-based diagnostic criteria were allowed as paraclinical evidence.

- Autopsy proven
- Definite
    1) Meet all Schumacher criteria
- Probable/strong possible
    1) Where case cannot be convincingly be made for satisfying all the Schumacher criteria
- Poor possible
    1) Where MS diagnosis not really indicated but where full exclusion of case as not-MS cannot be justified and a better explanation for symptoms/history cannot be made.
- Not MS
- Unknown

Appendix 3B.5.10 German Poser: 1973,Gottingen, Germany(25-27)

S. Poser and colleagues developed a system of diagnosis using optical mark forms which included data on a variety of clinical elements, including neurological symptoms and clinical history, the inputted data at the judgement of the neurologist. These factors were read by an optical mark form reader and patients allocated to:

- **Clinically-definite MS**
- **Probable MS**
- **Possible MS**

Appendix 3B.5.11 Borri Criteria: 1976, Italy(28)

- **Clinically-definite MS**

1) Patients in whom the diagnosis cannot be doubted, given their "typical" onset, disease course and symptoms at presentation.

- **Probable MS**

1) Patients with typical onset and clinical course, but no symptoms at presentation

- **Possible MS**

1) Patients with an atypical onset, course and/or symptoms, but in whom another explanation cannot be proved.

Appendix 3B.5.12 Rose Criteria: 1976(29)

- **Clinically-definite MS**

1) Relapsing-remitting course with at least two bouts separated by no less than one month

or

Slow or stepwise progressive course extending over at least 6-months.

2) Documented neurologic signs attributable to more than one site of predominantly white matter CNS pathology

3) Onset of symptoms usually between 10-50yo, inclusive

4) No better neurologic explanation

- **Probable MS**

1) History of relapsing-remitting symptoms but without documentation of signs and presenting with only one neurologic sign commonly associated with MS

or

Documented single bout of symptoms with signs of multifocal white matter disease with good recovery and followed by variable symptoms and signs.

2) No better neurological explanation

- **Possible MS**

1) History of relapsing-remitting symptoms without documentation or signs

or

Objective neurologic signs insufficient to establish more than one site of CNS white matter pathology

2) No better neurological explanation

Appendix 3B.5.13 Hader criteria: 1977, Saskatoon, Sask. Canada(30)

Criteria developed from Allison & Millar criteria, with a category for probable MS derived from the Schumacher criteria:

- Probable (clinically-definite)
    - Objective abnormalities on neurological examination attributable to dysfunction of the CNS; symptoms alone are not sufficient for a diagnosis.
    - At neurological exam or in medical history, there must be evidence of involvement in 2 or more separate parts of the CNS
    - Objective evidence of CNS disease must be predominantly of the white matter, with more than minor gray matter involvement disqualifying.
    - Involvement of the neuraxis must have occurred temporally in one of the following patterns:
        - 2 or more episode of worsening [relapse], separate by a period of one month or more, each episode lasting at least 24hrs.
        - Slow or step-wise progression of signs and symptoms over at least 6-months.
    - The age of the patient must be within 10-50yrs.
    - The signs and symptoms cannot be explained better by another disease process(21).
- Possible

- Includes cases in which there is some doubt as to the history or symptom evaluation, or there were other unusual or atypical features

- Suspect
  - Includes cases which primarily had a single neurologic episode, for which there was insufficient information, that were not referred to a neurologist for diagnosis, or those in which the patient refused interview/examination

Appendix 3B.5.14 McDonald-Halliday Criteria: 1977(31)

- **Proven MS**

1) diagnosis at necroscopy

- **Clinically definite MS**

1) All of the following:
   - RRMS history with two of more episodes [relapse]
   - Evidence of lesions at two or more necessarily separate sites in the CNS
   - Lesions predominantly in the white matter
   - Age at symptom onset between 10-50yo
   - History of signs and symptoms for at least one year
   - No better explanation for the observed abnormalities

- **Early probable or latent MS**

1) Single episode suggestive of MS

&

2) Evidence of lesions at two or more necessarily separate sites in the CNS

OR

3) Relapsing/remitting course

&

4) Evidence of only one lesion associated with MS

- **Progressive probable MS**

1) All of the following:

    - Progressive history of paraplegia
    - Evidence of lesions at two or more necessarily separate sites in the CNS
    - Other causes excluded

- **Progressive possible MS**

1) All of the following:

    - Progressive history of paraplegia
    - Evidence of only one lesion
    - Other causes excluded

- **Suspected MS**

1) Single episode suggestive of MS without evidence of any lesion or evidence of a single lesion only

OR

2) Recurrent optic neuritis (unilateral or bilateral) with one additional episode not involving the optic nerve but without evidence of lesions outside the eye

Appendix 3B.5.15 Numerical Poser criteria: 1979(32)

Poser proposed a system of classifying cases to probable and definite categories on the basis of a number of factors which were allotted points, with the total number of points present allowing the allocation of cases to a diagnostic group:

	Points
Age at MS symptom onset (years)	
10-19	1
20-29	3
30-39	4
40-49	1
First symptom (where multiple symptoms occur, highest scoring symptom should be chosen)	
Weakness (refers to specific pareses, not generalized fatigue; includes facial and/or limb weakness)	4
Ocular (refers to any and all signs/symptoms involving visual/oculomotor systems, including loss or diminution of visual acuity, blurring of vision, hemianopsia, double vision, ptosis, or loss of colour vision)	3
Paresthesiae (symptoms described by patients as numbness, pins & needles, formication, Lhermitte's sign; pain symptoms including trigeminal neuroglial and tabetics-like pains may be included as well)	2
Cerebellar (disturbance of equilibrium or difficulties in coordination)	1

Appendix 3B. Outline of diagnostic criteria utilised by studies included in meta-analysis, organised in approximate temporal order of publication.

Signs and symptoms at exam

Remission 7

(defines as the *complete* disappearance for at least one month of symptoms which had previously lasted at least 24hours)

Ocular 9

(refers to any and all signs/symptoms involving visual/oculomotor systems, including loss or diminution of visual acuity, blurring of vision, hemianopsia, double vision, ptosis, or loss of colour vision, as well as evidence of optic atrophy, abnormalities of flash/pattern reversal or alteration of colour vision testing)

Nystagmus 7

(also includes positive results of electronystagmogrpahy)

Weakness 10

(scored regardless of the number of limbs involved; scored if it is a patient complaint or determined by testing; also includes facial muscle weakness)

Spasticity/hyperreflexia 10

(scored regardless of the number of limbs involved; hyperreflexia must be definite; also includes sustained clonus or unilateral transient clonus)

Babinski sign 9

(scored even if present on one side only)

Absent abdominal reflex 8

(scored only if reflexes are absent on one or both sides; does not include easy fatigability of abdominal reflexes)

Appendix 3B. Outline of diagnostic criteria utilised by studies included in meta-analysis, organised in approximate temporal order of publication.

Gait ataxia	6
(scored only when disturbance represents cerebellar ataxia, as determined by history/examination)	
Incoordination	8
(should clearly reflect cerebellar involvement, including intention tremor, dysmetria, disorganization of movement, etc.)	
Dysarthria	6
(includes scanning speech or other articulatory disturbance but not cortical, aphasic problems; speech content should be normal)	
Urinary disturbance	8
(refers to frequency, urgency and stress incontinence and urinary retention; similar problems involving the bowel may also be scored)	
Paresthesiae	7
Diminished vibratory sense	6
Diminished position sense	6
Diminished pain sense	5
Mental changes	5
(scored only if the observed mental changes exceed the commonly observed reactive depression seen with MS; only blatant euphoria should be scored; evidence of cognitive impairment, either clinically or by psychological testing, should be obtained; also includes signs of dementia	

Appendix 3B.6 Poser criteria & modifications

Appendix 3B.6.1 Poser criteria: 1983(33)

- **A) Clinically-definite MS**
    1) Two attacks each lasting at least 24hrs, affecting different parts of the CNS and clinically evidence of two separate lesions
    2) Two attacks each lasting at least 24hrs, affecting different parts of the CNS; clinical evidence of one lesion and paraclinical evidence of another separate lesion
- **B) Laboratory-supported definite MS** (IgG oligoclonal bands in the CSF or increased IgG synthesis in the CNS, with normal levels of IgG in the serum)
    1) Two attacks each lasting at least 24hrs, affecting different parts of the CNS; either clinical or paraclinical evidence of one lesion; CSF oligoclonal bands or elevated IgG in the CNS
    2) One attack; clinical evidence of two separate lesions; CSF oligoclonal bands or elevated IgG in the CNS
    3) One attack; clinical evidence of one lesion and paraclinical evidence of another, separate lesion; CSF oligoclonal bands or elevated IgG in the CNS
- **C) Clinically-probable MS**
    1) Two attacks each lasting at least 24hrs, affecting different parts of the CNS and clinical evidence of one lesion
    2) One attack and clinical evidence of two separate lesions
    3) One attack; clinical evidence of one lesion and paraclinical evidence of another, separate lesion
- **D) Laboratory-supported probable MS**

1) Two attacks, each lasting at least 24hrs, affecting different parts of the CNS and CSF oligoclonal bands or elevated IgG in the CNS

Appendix 3B.6.2 Paty modifications to Poser criteria: 1988

The Paty requirements for dissemination in space by MRI diagnostic for MS were:

- At least 4 lesions at least 3mm in diameter

or

- 3 lesions, of which at least one is periventricular(34).

Appendix 3B.6.3 Fazekas modifications to Poser criteria: 1988(35)

Fazekas requirements for diagnosis of clinically-definite MS using MRI:

3 or more lesions with 2 of the following 3 properties:

- An infratentorial lesion
- A periventricular lesion
- Any lesions >6mm in diameter(35)

Appendix 3B.6.4 Barkhof modifications to Poser criteria: 1997(36)

Barkhof requirements for diagnosis of clinically-definite MS using MRI:

- At least one gadolinium-enhancing lesion or at least 9 T2-hyperintense lesions
- At least one intratentorial lesion
- At least one juxtacortical lesion
- At least three periventricular lesion

Appendix 3B.6.5 Chancellor modifications to Poser criteria: 2003, northern New Zealand(37)

Requirements for clinically-definite MS:

i) At least two attacks typical of a demyelinating episode (symptoms lasting at least 36hours) and neurological examination evidence of two lesions

or

ii) At least two attacks, examination evidence of one lesion and a MRI scan with more than three white-matter abnormalities (combination of axial and saggital T2 and FLAIR), at least one lesion periventricular

or

iii) One relapse of MS, clinical evidence of one lesion and a positive MRI, with new MR lesions developing over time

or

iv) Primary progressive myelopathy with a negative cranial MRI

Appendix 3B.7 McDonald Criteria: 2001(38)

Table 3. Diagnostic Criteria

Clinical Presentation	Additional Data Needed for MS Diagnosis
Two or more attacks; objective clinical evidence of 2 or more lesions	None[a]
Two or more attacks; objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by MRI[b] *or* Two or more MRI-detected lesions consistent with MS plus positive CSF[c] *or* Await further clinical attack implicating a different site
One attack; objective clinical evidence of 2 or more lesions	Dissemination in time, demonstrated by MRI[d] *or* Second clinical attack
One attack; objective clinical evidence of 1 lesion (monosymptomatic presentation; clinically isolated syndrome)	Dissemination in space, demonstrated by MRI[b] *or* Two or more MRI-detected lesions consistent with MS plus positive CSF[c] *and* Dissemination in time, demonstrated by MRI[d] *or* Second clinical attack
Insidious neurological progression suggestive of MS	Positive CSF[c] *and* Dissemination in space, demonstrated by 1) Nine or more T2 lesions in brain *or* 2) 2 or more lesions in spinal cord, *or* 3) 4–8 brain plus 1 spinal cord lesion *or* abnormal VEP[e] associated with 4–8 brain lesions, or with fewer than 4 brain lesions plus 1 spinal cord lesion demonstrated by MRI *and* Dissemination in time, demonstrated by MRI[d] *or* Continued progression for 1 year

If criteria indicated are fulfilled, the diagnosis is multiple sclerosis (MS); if the criteria are not completely met, the diagnosis is "possible MS"; if the criteria are fully explored and not met, the diagnosis is "not MS."

[a]No additional tests are required; however, if tests [magnetic resonance imaging (MRI), cerebral spinal fluid (CSF)] are undertaken and are *negative*, extreme caution should be taken before making a diagnosis of MS. Alternative diagnoses must be considered. There must be no better explanation for the clinical picture.
[b]MRI demonstration of space dissemination must fulfill the criteria derived from Barkhof et al[6] and Tintoré et al[7] (see Table 1).
[c]Positive CSF determined by oligoclonal bands detected by established methods (preferably isoelectric focusing) different from any such bands in serum or by a raised IgG index.[14,15]
[d]MRI demonstration of time dissemination must fulfill the criteria listed in Table 2.
[e]Abnormal visual evoked potential of the type seen in MS (delay with a well-preserved wave form).[16]

Appendix 3B.8 Polman criteria – revisions to McDonald criteria: 2005(39)

Table 4. The 2005 Revisions to the McDonald Diagnostic Criteria for Multiple Sclerosis

Clinical Presentation	Additional Data Needed for MS Diagnosis
Two or more attacks[a]; objective clinical evidence of two or more lesions	None[b]
Two or more attacks[a]; objective clinical evidence of one lesion	Dissemination in space, demonstrated by: • MRI[c] *or* • Two or more MRI-detected lesions consistent with MS plus positive CSF[d] *or* • Await further clinical attack[a] implicating a different site
One attack[a]; objective clinical evidence of two or more lesions	Dissemination in time, demonstrated by: • MRI[e] *or* • Second clinical attack[a]
One attack[a]; objective clinical evidence of one lesion (monosymptomatic presentation; clinically isolated syndrome)	Dissemination in space, demonstrated by: • MRI[c] *or* • Two or more MRI-detected lesions consistent with MS plus positive CSF[d] *and* Dissemination in time, demonstrated by: • MRI[e] *or* • Second clinical attack[a]
Insidious neurological progression suggestive of MS	One year of disease progression (retrospectively or prospectively determined) *and* Two of the following: a. Positive brain MRI (nine T2 lesions or four or more T2 lesions with positive VEP)[f] b. Positive spinal cord MRI (two focal T2 lesions) c. Positive CSF[d]

If criteria indicated are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is MS; if suspicious, but the criteria are not completely met, the diagnosis is "possible MS"; if another diagnosis arises during the evaluation that better explains the entire clinical presentation, then the diagnosis is "not MS."

[a]An attack is defined as an episode of neurological disturbance for which causative lesions are likely to be inflammatory and demyelinating in nature. There should be subjective report (backed up by objective findings) or objective observation that the event lasts for at least 24 hours.[1]

[b]No additional tests are required; however, if tests (MRI, CSF) are undertaken and are *negative*, extreme caution needs to be taken before making a diagnosis of MS. Alternative diagnoses must be considered. There must be no better explanation for the clinical picture and some objective evidence to support a diagnosis of MS.

[c]MRI demonstration of space dissemination must fulfill the criteria derived from Barkhof and colleagues[20] and Tintoré and coworkers[21] as presented in Table 2.

[d]Positive CSF determined by oligoclonal bands detected by established methods (isoelectric focusing) different from any such bands in serum, or by an increased IgG index.[56–58]

[e]MRI demonstration of time dissemination must fulfill the criteria in Table 1.

[f]Abnormal VEP of the type seen in MS.[59,60]

MS = multiple sclerosis; MRI = magnetic resonance imaging; CSF = cerebrospinal fluid; VEP = visual-evoked potential.

Appendix 3B.9 References

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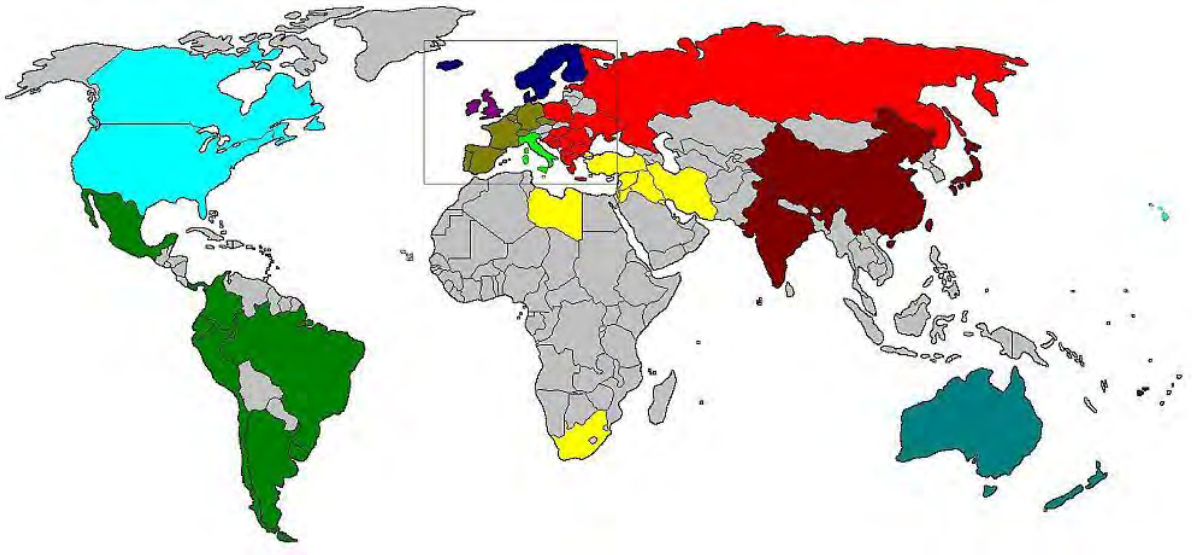
Appendix 3C. Regional allocation rationales.

In establishing the regions used for the regional sub-analyses, some classifications were simple, based largely upon physical boundaries and geographic proximity, while others were more complex, grouped together on the basis of cultural/historical reasoning, or genetic homogeneity. Some of the classifications, particularly the separate groups for the UK and Scandinavia from the rest of Western Europe, are distinct from some studies(1, 2) but are in harmony with others(3-6), and are to our thinking appropriate divisions of populations which are particularly distinct, both genetically and culturally. Similarly our division of the Italian region from the rest of Western Europe is novel from some studies(2-5) but is similar to grouping done elsewhere(1, 6) – here again, this division is appropriate, given the distinct epidemiology noted in the region(7-15), but also because of the novel inverse gradient in this region found in our analysis.

The regions will be discussed in a different order from that used in the body of the text, saving the regions of Europe for last, given the interrelatedness of the decision-making for these. Regions at the global level are illustrated in Map 1. Regions within Europe are illustrated in Map 2.

Appendix 3C. Figure 1. Map 1. World map showing the 10 study regions

aqua=Australasia; fuchsia=UK region; dark blue=Scandinavia & North Atlantic; green-brown=Atlantic & Central Europe; bright green=Italian region; red=Eastern Europe region; light blue=North America; green=Latin America & the Caribbean region; yellow=Middle East & Africa region; maroon=Asia & Pacific Islands region; grey=nations/regions without published prevalence data. Square boundary demarcates Europe region shown in Map 2.



Appendix 3C.2 Australasia region (aqua)

The Australasia region was demarcated as its own region, in keeping with virtually all reviews and meta-analyses which divided analyses into regions(2-6). The logic behind this is principally a matter of geography and genetics – the location of the two nations of Australia and New Zealand naturally suggests their grouping, and their genetic and cultural distinctiveness from the surrounding region precludes their being grouped with others.

Appendix 3C.3 Asia & Pacific Islands region (maroon)

This region was designated on the basis of the significant genetic and cultural differences between the nations of Asia and the Pacific Islands, here including Fiji, India, Japan, the People's Republic of China, and the Republic of China (Taiwan), and the rest of the world and this grouping is largely identical to that used elsewhere(2-6).

Appendix 3C.4 North America region (light blue)

The North America region was defined solely as the nations of Canada and the United States of America (USA), in contradistinction to previous works(3, 4) which also included Mexico and the nations of the Caribbean, though in keeping with others(2, 6). We decided to evaluate the North America region as Canada and the USA alone given the pattern of founding and resultant genetic/cultural characteristics of the nations of the Americas. Canada and the USA have largely British foundations, as well as large components of French-descent, while Central and South America were almost entirely colonized by the Spanish and Portuguese. The south-western areas of the USA (Colorado, New Mexico, southern California, Texas) have significant influence from Latin America, not merely by virtue of their former inclusion in the Spanish Empire and subsequent independent nation of Mexico, but also the modern-day immigration from Latin America. However the predominantly-American culture of these areas and significant non-Hispanic populations therein, as well as a desire to maintain regional divisions at the national-level wherever possible left these to be included with the rest of the USA and thus, with the North America region.

Appendix 3C.5 Latin America and Caribbean region (green)

This regional definition follows from the preceding North America region, encompassing the other nations of the Americas in Central and South America, and the Caribbean: Argentina, Brazil, Chile, Colombia, Ecuador, the French West Antilles of France, Mexico, Panama, Peru and Uruguay. As noted above, the nations of Central and South America are linked by their colonization by Spain and Portugal, giving them distinct genetic and cultural characteristics from the rest Canada and the USA, alongside significant homology with one another. The major exception to this Hispanic descent is in the French West Antilles, an overseas department of France. The significant non-European population, largely of African descent, renders this nation distinct from both North America and Latin America. However, on the basis of geography, and previous studies[35,36], it was decided to include the French West Antilles in the grouping with Latin America.

Appendix 3C.6 Middle East & Africa region (yellow)

Here again, the distinctiveness of the populations on the basis of genetic and cultural factors necessitate the separation of the nations in this region, Iran, Iraq, Israel, Jordan, Kuwait, Libya, Malta, Qatar, Saudi Arabia, South Africa, and Turkey, from their neighbours in Europe and Asia. While naturally the nations of the Middle East, Malta and Libya are quite distinct from South Africa, it was nonetheless necessary given the paucity of prevalence estimates in the latter. The two populations are not irreconcilable and certainly the populations of South Africa are not dissimilar from the nations of North Africa, and the latter are culturally linked to the nations of the Middle East.

This grouping is similar to that used elsewhere(2-5), but is distinct in that we have included Turkey with the Middle East, rather than Europe. Turkey is a bridge between Europe and the Middle East, both physically and to varying extent, genetically and culturally. It was decided however that the ties binding Turkey to the Middle East were stronger, by virtue of the much greater cultural homology between these two(16) than between Turkey and the Eastern Europe region, with which Turkey would otherwise be grouped, as well as by racial/genetic groupings, again with much greater homology between Turkey and the Middle East, than with Eastern Europe.

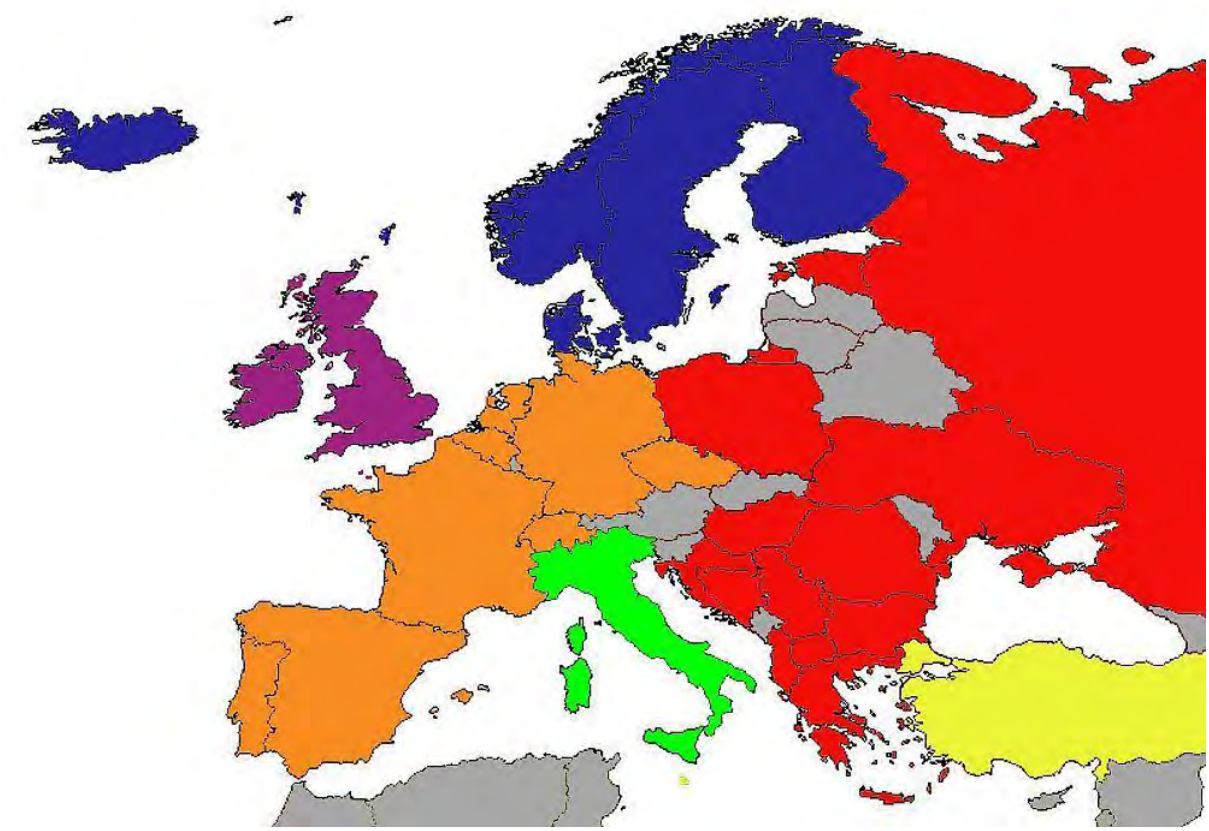
A further significant change from the allocations found elsewhere is our inclusion of the nation of Malta with the Middle East & Africa region. While the Maltese population shows some homology with Sicily and Italy(17), other work indicates a greater influence from the other side of the Mediterranean, in Tunisia, and directly from Phoenicia(18). Certainly this hypothesis is borne out better by history, wherein Malta was, like Carthage, a major Phoenician settlement(16, 18). A further indication is that the inclusion of Malta in the analysis with the Italian region abrogates the strong inverse association observed there, suggesting a different relationship in Malta from Italy and Corsica.

Appendix 3C.7 Europe

In evaluating Europe, we sought to take into account genetic and cultural differences to a much greater extent than geography, which necessarily becomes less prominent in the smaller area in question. An additional factor was the distribution of prevalence estimates – given the much greater number of studies done in Europe, finer divisions could be made to divide the populations more properly in consideration of their historical and genetic divisions, as well as to better capture unique associations which were present in the different regions. While we did evaluate the whole of Western Europe in keeping with work elsewhere(2), we also evaluated the regions, demonstrating the presence of independent and distinct epidemiological associations which would otherwise be obscured in the aggregate due to an "averaging" of the individual effects.

Appendix 3C. Figure 2. Map 2. Inset area from Figure 1 showing Europe and its constituent study regions

fuchsia=UK region; dark blue=Scandinavia & North Atlantic; orange=Atlantic & Central Europe region; bright green=Italian region; red=Eastern Europe; yellow=Middle East & Africa region; grey=nations/regions with no prevalence data.



Appendix 3C.7.1 United Kingdom & Ireland region (fuchsia)

We chose to evaluate the UK & Ireland separate from the rest of Western Europe, not merely for the closer genetic homology of these two island nations, but the cultural and historical differences from mainland Europe afforded by their physical separation(16). This separation is in keeping with previous work(3-6) though some studies condensed this region along with the rest of Western Europe(1, 2), which is to our minds inappropriate.

Appendix 3C.7.2 Scandinavia & North Atlantic (dark blue)

This region, including the Scandinavian nations of Denmark, Finland, Norway and Sweden, as well as Iceland, the Faroe Islands of Denmark, and the Shetland Islands of the UK was analysed separate from the rest of Western Europe given the homology of the nations of Scandinavia, and including the island nations as a consequence of their shared Nordic history(16). While this grouping is in keeping with previous work(3-6), albeit not all(1, 2), the inclusion of the Shetland Islands is distinct from all previous studies.

We chose to include the Shetlands with the Nordic nations, rather than with the UK and Ireland, given the lasting influences of Norwegian rule on the population(19, 20), and indeed, its population remains largely descended from the original Norwegian settlers(21), likely a consequence of the physical remoteness of the Shetland Islands from mainland Scotland. This is in contradistinction from Orkney, which despite also being under Norwegian rule at the same time as Shetland, is much closer genetically to Scotland than Scandinavia than is Shetland(22), possibly due to its much closer proximity to Scotland.

An argument could have been made for excluding Finland from the Scandinavian analysis. While certainly they are geographically and culturally related, the population of Finland are genetically quite distinct from the other Scandinavian nations(23) and the rest of Western Europe(24), representing a complex genetic mixture due to their location between Western and Eastern Europe(25). However, given the aforementioned geographic and cultural links, as well as the difficulty of then placing Finland, and particularly, given the population mixing found in the northern latitudes of Sweden, Norway and Finland, where all populations include some component of Finn(26, 27), we elected to leave Finland with the rest of the Scandinavia & North Atlantic region.

Appendix 3C.7.3 Italian region (bright green)

This region was defined due to the need to evaluate Italy independently from the rest of Europe, as well as geographical considerations, and is in keeping with some previous reviews(1, 6). Italy has some

genetic homology with Spain and Portugal(28) and the expansionism of the Roman Empire had lasting influences on the culture and language of much of Western Europe(16). However the unique epidemiology of MS in the Italian region(7-15), particularly Sardinia, necessitated its evaluation independently.

The inclusion of peninsular and insular Italy necessitated the additional inclusion of the island of Corsica. While Corsica is part of France, this only goes back to the mid 18th century(16) and indeed genetic analysis shows that Corsicans are more closely related to Sardinians than to the French(29), which is to be expected given the history, as well as the physical proximity.

Appendix 3C.7.4 Eastern Europe (red)

This region was demarcated on both genetic and historical-cultural bases, the latter being principally defined as those nations which were under Soviet influence over much of the 20th century(16) (excepting East Germany, which was evaluated with West Germany, and the Czech Republic, which was also evaluated with Germany) and is in keeping with work elsewhere(2). This region thus included Albania, Bosnia-Herzegovina, Bulgaria, Croatia, Estonia, Greece, Hungary, Lithuania, Macedonia, Poland, Romania, Russia, Serbia, Ukraine, and the country formerly known as Yugoslavia. While the nations in the region are naturally quite distinct, both genetically and culturally, encompassing a huge swath of peoples and territory, many are linked genetically(28) and share linguo-cultural systems of similar derivation(16).

Appendix 3C.7.5 Atlantic & Central Europe (orange)

This grouping was difficult, given the paucity of age-standardisable prevalence estimates in the two major constituent regions comprised of those nations on the coast of the Atlantic Ocean and English Channel – Belgium, France, the Netherlands, Portugal and Spain - and the Germanic nations – the Czech Republic, Germany, and Switzerland. The former share a number of linguo-cultural links inherent in their shared histories and physical proximity(16), as well as some genetic overlap(28, 30-

32). The latter are thoroughly intertwined, both genetically(28, 30-32) as well as in their language, culture and history. Unfortunately, as it was desirous to be able to evaluate associations after age-standardisation, these two regions had to be consolidated. The two are not uniformly dissimilar however – France shows some genetic homology with the Germanic region(28), and continental Western Europe shares a number of cultural and historical interactions(16).

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Appendix 3D. HLA-DR frequencies utilised for each of study locations for which data could be obtained.

Data for each prevalence study's HLA-DRB1 allele frequencies, where data is available. Includes alleles associated with increased risk (*15, *03) and those associated with reduced risk (*01, *11, *14). All HLA-DRB1 allele frequency data obtained from online databases, or individual publications, as specified in Methods.

		HLA-DRB1 allele frequencies (%)				
Study area	Latitude	*15	*03	*01	*11	*14
Appendix 3D.1 United Kingdom of Great Britain & Northern Ireland and Republic of Ireland						
Bailiwick of Guernsey, UK	49.50	18.3	11.6	12.6	4.3	3.6
Bailiwick of Jersey, UK	49.50	18.3	11.6	12.6	4.3	3.6
Brighton & Mid-Downs Health Districts, England, UK	50.08	14.7	15.3	12.6	5.9	2.8
Cornwall, England, UK	50.30	13.8	16.6	8.7	5.3	2.4
Devon, England, UK	50.70	13.8	16.6	8.7	5.3	2.4
Southampton/ Southwest Hampshire Health Authority, England, UK	50.90	13.8	16.6	8.7	5.3	2.4
Sutton borough, South London, England, UK	51.33	14.7	15.3	12.6	5.9	2.8
Cardiff unitary authority, Wales, UK	51.50	13.8	16.6	8.7	5.3	2.4
South Glamorgan County, Wales, UK	51.67	13.8	16.6	8.7	5.3	2.4
South Cambridgeshire, England, UK	52.19	14.7	15.3	12.6	5.9	2.8
Cambridge Health District, England, UK	52.38	14.7	15.3	12.6	5.9	2.8
Suffolk County, England, UK	52.43	14.7	15.3	12.6	5.9	2.8
North Cambridgeshire, England, UK	52.58	14.7	15.3	12.6	5.9	2.8
County Wexford, Republic of Ireland	52.77	13.8	18.5	12.8	5.0	3.5
Bradford, England, UK	52.80	14.7	15.3	12.6	5.9	2.8
Carlisle, England, UK	52.89	15.0	15.3	11.3	4.8	2.1
Bassetlaw Health District, England, UK	53.00	14.7	15.3	12.6	5.9	2.8
Republic of Ireland	53.43	19.0	17.0	7.0	1.8	
Rochdale, Manchester, England, UK	53.62	14.7	15.3	12.6	5.9	2.8
Leeds Health Authority, England, UK	53.80	14.8	14.6	12.6	6.1	2.2
Northeast Ireland, Republic of Ireland	54.58	19.0	17.0	7.0	1.8	
Durham County, Scotland, UK	54.70	14.7	15.3	12.6	5.9	2.8
County Donegal, Republic of Ireland	54.92	19.7	17.5	11.5	4.0	1.0

Appendix 3D. HLA-DR frequencies utilised for each of study locations for which data could be obtained.

Northern Ireland, UK	55.00	18.6	16.0	12.0	2.4	1.5
Northumberland County, Scotland, UK	55.34	14.7	15.3	12.6	5.9	2.8
Lothian & Border Health Board Regions, Scotland, UK	55.75	14.7	15.3	12.6	5.9	2.8
Glasgow, Scotland, UK	55.86	14.7	15.3	12.6	5.9	2.8
Fife, Scotland, UK	56.22	14.7	15.3	12.6	5.9	2.8
Tayside Health Board, Scotland, UK	56.70	14.7	15.3	12.6	5.9	2.8
Northeast Scotland, UK	57.22	14.7	15.3	12.6	5.9	2.8
County of Inverness, Scotland, UK	57.47	14.7	15.3	12.6	5.9	2.8
County of Nairn, Scotland, UK	57.58	14.7	15.3	12.6	5.9	2.8
Ross & Cromarty County, Scotland, UK	57.66	14.7	15.3	12.6	5.9	2.8
Outer Hebrides, Scotland, UK	57.72	14.7	15.3	12.6	5.9	2.8
County of Sutherland, Scotland, UK	58.25	14.7	15.3	12.6	5.9	2.8
County of Caithness, Scotland, UK	58.42	14.7	15.3	12.6	5.9	2.8
Orkney Islands, Scotland, UK	59.00	18.1	10.2	5.3	2.4	1.9

Appendix 3D.2 Scandinavia and North Atlantic

Kingdom of Sweden	55.78	31.0	0.4	20.0	13.0	5.1
Kingdom of Denmark	56.27	17.6	10.2	13.0	0.9	0.9
Kingdom of Norway	59.43	17.0	14.0	11.6	4.0	3.0
Stockholms län, Kingdom of Sweden	59.50	18.1	10.2	5.3	2.4	1.9
Republic of Finland	60.38	8.3	2.8	6.1	0.6	1.1
Shetland Islands, Scotland, UK	60.50	18.1	10.2	5.3	2.4	1.9

Appendix 3D.3 Atlantic & Central Europe

La Palma Island, Islas Canarias, Kingdom of Spain	28.10	11.0			15.0	
Las Palmas, Islas Canarias, Kingdom of Spain	28.22	11.0			15.0	
Lanzarote, Las Palmas, Islas Canarias, Kingdom of Spain	29.04	11.0			15.0	
Vélez-Málaga Sanitary District, Kingdom of Spain	36.77	11.3	13.7	10.2	13.7	2.5
Santarém, Portuguese Republic	39.20	8.0	11.5	12.3	10.6	2.8
Móstoles, Kingdom of Spain	40.32	14.0	13.4	11.7	12.8	
Calatayud Sanitary District, Kingdom of Spain	41.31	14.6	7.3	16.9	5.1	2.2
Valladolid, Kingdom of Spain	41.65	14.6	7.3	16.9	5.1	2.2
Zaragoza, Kingdom of Spain	41.66	14.6	7.3	16.9	5.1	2.2
Osona, Kingdom of Spain	41.83	14.6	7.3	16.9	5.1	2.2
Huesca, Kingdom of Spain	42.13	14.6	7.3	16.9	5.1	2.2

Appendix 3D. HLA-DR frequencies utilised for each of study locations for which data could be obtained.

Hautes-Pyrénées département, French Republic	42.50	13.9	13.1	8.6	13.1	3.7
Comunidad Foral de Navarra, Kingdom of Spain	42.62	14.6	7.3	16.9	5.1	2.2
Gijón Health District, Asturias, Kingdom of Spain	43.00	14.6	7.3	16.9	5.1	2.2
Gijón, Asturias, Kingdom of Spain	43.00	14.6	7.3	16.9	5.1	2.2
Marseille, French Republic	43.25	13.9	13.1	8.6	13.1	3.7
Haute-Garonne, French Republic	43.31	13.9	13.1	8.6	13.1	3.7
Midi- Pyrénées Région, French Republic	43.50	13.9	13.1	8.6	13.1	3.7
Languedoc -Roussillon Région, French Republic	43.67	8.0	9.7	11.0	8.4	5.1
Avignon, French Republic	44.00	8.0	9.7	11.0	8.4	5.1
Provence-Alpes-Côte d'Azur Région, French Republic	44.00	8.0	9.7	11.0	8.4	5.1
Aquitaine Région, French Republic	44.58	13.9	13.1	8.6	13.1	3.7
Auvergne Région, French Republic	45.33	13.9	13.1	8.6	13.1	3.7
Rhône-Alpes Région, French Republic	45.36	8.0	9.7	11.0	8.4	5.1
Limousin Région, French Republic	45.69	13.9	13.1	8.6	13.1	3.7
Poitou-Charentes Région, French Republic	46.08	13.9	13.1	8.6	13.1	3.7
Chalon-sur-Saône, French Republic	47.00	9.7	11.3	10.8	15.0	3.7
Bourgogne Région, French Republic	47.00	18.3	11.6	12.6	4.3	3.6
Franche-Comté Région, French Republic	47.00	9.7	11.3	10.8	15.0	3.7
Côte-d'Or département, French Republic	47.42	9.7	11.3	10.8	15.0	3.7
Pays de la Loire Région, French Republic	47.47	13.3	15.2	7.0	6.8	4.0
Centre Région, French Republic	47.50	9.7	11.3	10.8	15.0	3.7
Morbihan département, French Republic	47.83	13.3	15.2	7.0	6.8	4.0
Santiago de Compostela, Kingdom of Spain	47.98	9.6	12.8	11.5	12.6	2.6
Bretagne Région, French Republic	48.00	9.7	11.3	10.8	15.0	3.7
Ille-et-Vilaine département, French Republic	48.17	13.3	15.2	7.0	6.8	4.0
Finistère département, French Republic	48.25	18.3	11.6	12.6	4.3	3.6
Côtes-d'Armor département , French Republic	48.33	18.3	11.6	12.6	4.3	3.6
Alsace Région, French Republic	48.50	9.7	11.3	10.8	15.0	3.7

Appendix 3D. HLA-DR frequencies utilised for each of study locations for which data could be obtained.

Île-de-France Région, French Republic	48.50	9.7	11.3	10.8	15.0	3.7
Bas-Rhin département , French Republic	48.58	9.7	11.3	10.8	15.0	3.7
Lorraine Région, French Republic	49.00	9.7	11.3	10.8	15.0	3.7
Basse-Normandie Région, French Republic	49.00	9.7	11.3	10.8	15.0	3.7
Champagne-Ardenne Région, French Republic	49.00	9.7	11.3	10.8	15.0	3.7
Jihocesky kraj, Czech Socialist Republic	49.06	11.0	7.1	7.6	6.7	3.8
Jihomoravsky kraj, Czech Socialist Republic	49.09	11.0	7.1	7.6	6.7	3.8
Haute-Normandie Région, French Republic	49.50	9.7	11.3	10.8	15.0	3.7
Picardie Région, French Republic	49.50	9.7	11.3	10.8	15.0	3.7
Severomoravský kraj, Czech Socialist Republic	49.57	11.0	7.1	7.6	6.7	3.8
Západočeský kraj, Czech Socialist Republic	49.70	11.0	7.1	7.6	6.7	3.8
46 communities in and around Spessart, Federal Republic of Germany	49.88	14.6	9.0	11.1	7.4	3.1
Darmstadt, Federal Republic of Germany	49.98	14.6	9.0	11.1	7.4	3.1
Southern Hesse, Federal Republic of Germany	49.98	14.6	9.0	11.1	7.4	3.1
Středočeský kraj, Czech Socialist Republic	50.06	11.0	7.1	7.6	6.7	3.8
Východočeský kraj, Czech Socialist Republic	50.19	11.0	7.1	7.6	6.7	3.8
Nord-Pas-de-Calais Région, French Republic	50.47	9.7	11.3	10.8	15.0	3.7
Praha oblast, Czech Socialist Republic	50.55	11.0	7.1	7.6	6.7	3.8
Severočeský kraj, Czech Socialist Republic	50.57	11.0	7.1	7.6	6.7	3.8
Okres Teplice, Czech Republic	50.60	11.0	7.1	7.6	6.7	3.8
Leuven, Flemish Region, Kingdom of Belgium	50.88	14.2	15.7	8.6	7.1	1.5
Bezirk Halle, German Democratic Republic	51.00	14.6	9.0	11.1	7.4	3.1
Göttingen, Federal Republic of Germany	51.77	14.6	9.0	11.1	7.4	3.1
Southern Lower Saxony, Federal Republic of Germany	52.02	14.6	9.0	11.1	7.4	3.1
Provincie Groningen, Kingdom of the Netherlands	53.17	9.3	11.6	6.6	8.3	3.8
Freie und Hansestadt Hamburg, Federal Republic of Germany	53.75	14.6	9.0	11.1	7.4	3.1
German Democratic Republic	54.10	14.6	9.0	11.1	7.4	3.1
Rostock-Stadt, Rostock-Land & Bad Doberan Landkreise, German Democratic Republic	54.12	14.6	9.0	11.1	7.4	3.1
Stralsund region, German Democratic Republic	54.30	14.6	9.0	11.1	7.4	3.1

Appendix 3D.4 Italian region

Città di Agrigento, Sicilia, Italian Republic	37.32	10.8	10.8	6.0	17.5	3.0

Appendix 3D. HLA-DR frequencies utilised for each of study locations for which data could be obtained.

Comune di Caltanissetta, Sicilia, Italian Republic	37.45	10.8	10.8	6.0	17.5	3.0
Comune di Catania, Sicilia, Italian Republic	37.50	5.6	5.1	7.9	32.6	6.2
Comune di Enna, Sicilia, Italian Republic	37.56	5.6	5.1	7.9	32.6	6.2
Comune di Linguaglossa, Sicilia, Italian Republic	37.85	5.6	5.1	7.9	32.6	6.2
Provincia di Messina, Sicilia, Italian Republic	38.08	5.6	5.1	7.9	32.6	6.2
Città di Bagheria, Sicilia, Italian Republic	38.08	5.1	10.8	11.4	22.2	4.0
Comune di Monreale, Sicilia, Italian Republic	38.08	5.1	10.8	11.4	22.2	4.0
Southwest Sardegna, Italian Republic	39.33	4.6	55.7	3.0	1.5	0.2
Provincia di Cagliari, Sardegna, Italian Republic	39.37	4.6	55.7	3.0	1.5	0.2
Provincia di Nuoro, Sardegna, Italian Republic	40.20	4.6	55.7	3.0	1.5	0.2
Barbagia, Provincia di Nuoro , Sardegna, Italian Republic	40.32	4.6	55.7	3.0	1.5	0.2
Provincia di Salerno, Italian Republic	40.42	6.3	6.0	7.4	12.8	7.0
Città di Alghero, Sardegna, Italian Republic	40.48	4.6	55.7	3.0	1.5	0.2
Sorrento Peninsula, Campania, Italian Republic	40.64	4.7	6.6	4.8	29.0	7.6
Cava de' Tirreni, Campania, Italian Republic	40.70	4.7	6.6	4.8	29.0	7.6
Comune di Sassari, Sardegna, Italian Republic	40.73	4.6	55.7	3.0	1.5	0.2
Campania region, Italian Republic	40.75	4.6	55.7	3.0	1.5	0.2
Northwest Sardegna, Italian Republic	40.75	4.6	55.7	3.0	1.5	0.2
Provincia di Sassari, Sardegna, Italian Republic	40.75	4.7	6.6	4.8	29.0	7.6
Provincia di Napoli, Italian Republic	40.79	6.3	6.0	7.4	12.8	7.0
Comune di Napoli, Italian Republic	40.83	6.3	6.0	7.4	12.8	7.0
Comune di Nusco, Italian Republic	40.88	6.3	6.0	7.4	12.8	7.0
Provincia di Avellino, Italian Republic	40.97	6.3	6.0	7.4	12.8	7.0
Provincia di Bari, Italian Republic	40.98	6.3	6.0	7.4	12.8	7.0
Provincia di Caserta, Italian Republic	41.20	6.3	6.0	7.4	12.8	7.0
Provincia di Benevento, Italian Republic	41.26	6.3	6.0	7.4	12.8	7.0
Provincia dell'Aquila, Italian Republic	42.10	3.1	7.2	9.4	20.5	4.8
Corse département, French Republic:	42.15	13.9	13.1	8.6	13.1	3.7
Comune dell'Aquila, Italian Republic	42.33	3.1	7.2	9.4	20.5	4.8
Umbria region, Italian Republic	42.97	3.1	7.2	9.4	20.5	4.8
Republic of San Marino	43.93	3.1	7.2	9.4	20.5	4.8
Comune di Modena, Italian Republic	44.30	3.1	7.2	9.4	20.5	4.8
Reggio Emilia e Modena Province, Italian Republic	44.33	3.1	7.2	9.4	20.5	4.8

Appendix 3D. HLA-DR frequencies utilised for each of study locations for which data could be obtained.

Comune di Genova, Italian Republic	44.40	3.1	7.2	9.4	20.5	4.8
Provincia di Parme Italian Republic	44.70	3.1	7.2	9.4	20.5	4.8
Provincia di Ferrara, Italian Republic	44.75	3.1	7.2	9.4	20.5	4.8
Comune di Copparo, Italian Republic	44.90	3.1	7.2	9.4	20.5	4.8
Città di Pavia, Italian Republic	45.07	4.3	7.7	9.2	25.0	6.2
Città di Padova, Italian Republic	45.42	3.1	7.2	9.4	20.5	4.8
Provincia di Venezia, Italian Republic	45.47	3.1	7.2	9.4	20.5	4.8
Provincia di Novara, Italian Republic	45.63	3.1	7.2	9.4	20.5	4.8
Valle d'Aosta, Italian Republic	45.73	3.1	7.2	9.4	20.5	4.8
Provincia di Varese, Italian Republic	45.85	3.1	7.2	9.4	20.5	4.8

Appendix 3D.5 Eastern Europe

Western Greece, Hellenic Republic	38.29	8.2	7.0	6.3	27.7	5.2
Northern Greece, Hellenic Republic	40.79	6.8	7.4	6.8	27.8	4.8
Evros Prefecture, Hellenic Republic	41.08	6.8	7.4	6.8	27.8	4.8
Samokov, Republic of Bulgaria	42.33	5.4	8.2	6.4	23.7	1.8
Burgas, People's Republic of Bulgaria	42.50	5.4	8.2	6.4	23.7	1.8
Silven, People's Republic of Bulgaria	42.68	5.4	8.2	6.4	23.7	1.8
Sofia, People's Republic of Bulgaria	42.70	5.4	8.2	6.4	23.7	1.8
Ravno, Bosnia & Herzegovina	42.88	12.7	9.3	9.7	14.9	7.1
Trojan, Republic of Bulgaria	42.88	5.4	8.2	6.4	23.7	1.8
Neum, Bosnia & Herzegovina	42.92	12.7	9.3	9.7	14.9	7.1
Svoge, Republic of Bulgaria	42.97	10.9	10.8	8.8	17.6	6.4
Dubrovnik-Neretva županije, Republic of Croatia	42.97	10.9	10.8	8.8	17.6	6.4
Veliko Tirnovo, People's Republic of Bulgaria	43.03	5.4	8.2	6.4	23.7	1.8
Stolac, Bosnia & Herzegovina	43.08	12.7	9.3	9.7	14.9	7.1
Čapljina, Bosnia & Herzegovina	43.11	12.7	9.3	9.7	14.9	7.1
Čitluk, Bosnia & Herzegovina	43.20	12.7	9.3	9.7	14.9	7.1
Ljubuški, Bosnia & Herzegovina	43.20	12.7	9.3	9.7	14.9	7.1
Mostar, Bosnia & Herzegovina	43.33	12.7	9.3	9.7	14.9	7.1
Široki Brijeg, Bosnia & Herzegovina	43.37	12.7	9.3	9.7	14.9	7.1
Grude, Bosnia & Herzegovina	43.38	12.7	9.3	9.7	14.9	7.1
Mihaylovgrad, People's Republic of Bulgaria	43.42	5.4	8.2	6.4	23.7	1.8
Posušje,	43.47	12.7	9.3	9.7	14.9	7.1

Appendix 3D. HLA-DR frequencies utilised for each of study locations for which data could be obtained.

Bosnia & Herzegovina						
Konjic, Bosnia & Herzegovina	43.65	12.7	9.3	9.7	14.9	7.1
Jablanica, Bosnia & Herzegovina	43.66	12.7	9.3	9.7	14.9	7.1
Prozor-Rama, Bosnia & Herzegovina	43.82	12.7	9.3	9.7	14.9	7.1
Giurgiu judet, Socialist Republic of Romania	44.14	4.6	5.7	4.1	10.0	3.6
Dolj judet, Socialist Republic of Romania	44.21	4.6	5.7	4.1	10.0	3.6
Constanta judet, Socialist Republic of Romania	44.29	4.6	5.7	4.1	10.0	3.6
North Adriatic Islands, Republic of Croatia	44.38	4.6	5.7	4.1	10.0	3.6
Bucureşti, Romania	44.42	10.9	10.8	8.8	17.6	6.4
Ialomita judet, Socialist Republic of Romania	44.45	4.6	5.7	4.1	10.0	3.6
Prahova judet, Socialist Republic of Romania	44.99	4.6	5.7	4.1	10.0	3.6
Argeş judet, Socialist Republic of Romania	45.01	4.6	5.7	4.1	10.0	3.6
Tulcea judet, Socialist Republic of Romania	45.02	4.6	5.7	4.1	10.0	3.6
Vilcea judet, Socialist Republic of Romania	45.03	4.6	5.7	4.1	10.0	3.6
Braila judet, Socialist Republic of Romania	45.05	4.6	5.7	4.1	10.0	3.6
Istria, Socialist Republic of Croatia	45.19	4.6	5.7	4.1	10.0	3.6
Caras-Severin judet, Socialist Republic of Romania	45.21	10.9	10.8	8.8	17.6	6.4
Buzau judet, Socialist Republic of Romania	45.23	4.6	5.7	4.1	10.0	3.6
Calarasi judet, Socialist Republic of Romania	45.39	4.6	5.7	4.1	10.0	3.6
Northeast Istria, Republic of Croatia	45.39	4.6	5.7	4.1	10.0	3.6
Gorski kotar, Republic of Croatia	45.53	10.9	10.8	8.8	17.6	6.4
Timis judet, Socialist Republic of Romania	45.59	10.2	14.2	8.7	11.9	0.8
Brasov judet, Socialist Republic of Romania	45.76	4.6	5.7	4.1	10.0	3.6
Vrancea judet, Socialist Republic of Romania	45.79	4.6	5.7	4.1	10.0	3.6
Galati judet, Socialist Republic of Romania	45.80	4.6	5.7	4.1	10.0	3.6
Hunedoara judet, Socialist Republic of Romania	45.83	4.6	5.7	4.1	10.0	3.6
Sibiu judet, Socialist Republic of Romania	45.84	4.6	5.7	4.1	10.0	3.6
Covasna judet, Socialist Republic of Romania	45.89	4.6	5.7	4.1	10.0	3.6
Alba judet, Socialist Republic of Romania	46.00	4.6	5.7	4.1	10.0	3.6
Cluj judet, Socialist Republic of Romania	44.29	4.6	5.7	4.1	10.0	3.6
Varaždinska županija, Republic of Croatia	46.22	10.9	10.8	8.8	17.6	6.4
Arad judet, Socialist Republic of Romania	46.31	4.6	5.7	4.1	10.0	3.6
Bacau judet, Socialist Republic of Romania	46.42	4.6	5.7	4.1	10.0	3.6

Appendix 3D. HLA-DR frequencies utilised for each of study locations for which data could be obtained.

Vaslui judet, Socialist Republic of Romania	46.51	4.6	5.7	4.1	10.0	3.6
Harghita judet, Socialist Republic of Romania	46.61	4.6	5.7	4.1	10.0	3.6
Mureş judet, Socialist Republic of Romania	46.63	4.6	5.7	4.1	10.0	3.6
Cluj-Napoca, Socialist Republic of Romania	46.77	4.6	5.7	4.1	10.0	3.6
Iasi judet, Socialist Republic of Romania	46.88	4.6	5.7	4.1	10.0	3.6
Neamt judet, Socialist Republic of Romania	46.93	4.6	5.7	4.1	10.0	3.6
Bihor judet, Socialist Republic of Romania	46.95	4.6	5.7	4.1	10.0	3.6
Salaj judet, Socialist Republic of Romania	47.05	4.6	5.7	4.1	10.0	3.6
Bistriţa-Năsăud district, Socialist Republic of Romania	47.17	4.6	5.7	4.1	10.0	3.6
Sălaj judet, Socialist Republic of Romania	47.17	4.6	5.7	4.1	10.0	3.6
Bistriţa - Năsăud judet, Socialist Republic of Romania	47.22	4.6	5.7	4.1	10.0	3.6
Suceava judet, Socialist Republic of Romania	47.49	4.6	5.7	4.1	10.0	3.6
Maramureş judet, Socialist Republic of Romania	47.67	4.6	5.7	4.1	10.0	3.6
Judeţul Satu Mare, Socialist Republic of Romania	47.71	4.6	5.7	4.1	10.0	3.6
Botosani judet, Socialist Republic of Romania	47.78	4.6	5.7	4.1	10.0	3.6
Gorski kotar-Kočevje region, Republic of Croatia	48.27	10.2	14.2	8.7	11.9	0.8
Western Herzegovina, Bosnia and Herzegovina	48.27	12.7	9.3	9.7	14.9	7.1
Lublin, Republic of Poland	51.23	12.8	11.5	9.8	6.5	2.0
Kępno powiat, People's Republic of Poland	51.27	12.8	11.5	9.8	6.5	2.0
Ostrzeszów powiat, People's Republic of Poland	51.42	12.8	11.5	9.8	6.5	2.0
Środa powiat, People's Republic of Poland	51.44	12.8	11.5	9.8	6.5	2.0
Wolsztyn powiat, People's Republic of Poland	51.55	12.8	11.5	9.8	6.5	2.0
Ostrów Wielkopolski powiat, People's Republic of Poland	51.60	12.8	11.5	9.8	6.5	2.0
Rawicz powiat, People's Republic of Poland	51.67	12.8	11.5	9.8	6.5	2.0
Krotoszyn powiat, People's Republic of Poland	51.72	12.8	11.5	9.8	6.5	2.0
Kalisz powiat, People's Republic of Poland	51.76	12.8	11.5	9.8	6.5	2.0
Gostyń powiat, People's Republic of Poland	51.82	12.8	11.5	9.8	6.5	2.0
Leszno powiat, People's Republic of Poland	51.84	12.8	11.5	9.8	6.5	2.0
Pleszew powiat, People's Republic of Poland	51.92	12.8	11.5	9.8	6.5	2.0
Turek powiat, People's Republic of Poland	52.00	12.8	11.5	9.8	6.5	2.0
Kościan powiat, People's Republic of Poland	52.10	12.8	11.5	9.8	6.5	2.0
Pruszków, People's Republic of Poland	52.17	12.8	11.5	9.8	6.5	2.0

Appendix 3D. HLA-DR frequencies utilised for each of study locations for which data could be obtained.

Warsaw, People's Republic of Poland	52.23	12.8	11.5	9.8	6.5	2.0
Powiat Kolski, People's Republic of Poland	52.24	12.8	11.5	9.8	6.5	2.0
Września powiat, People's Republic of Poland	52.24	12.8	11.5	9.8	6.5	2.0
Śrem powiat, People's Republic of Poland	52.31	12.8	11.5	9.8	6.5	2.0
Słupca powiat, People's Republic of Poland	52.31	12.8	11.5	9.8	6.5	2.0
Nowy Tomyśl powiat, People's Republic of Poland	52.34	12.8	11.5	9.8	6.5	2.0
Jarocin powiat, People's Republic of Poland	52.40	16.2	7.6	9.6	6.6	0.5
Poznań, People's Republic of Poland	52.40	12.8	11.5	9.8	6.5	2.0
Poznań powiat, People's Republic of Poland	52.45	16.2	7.6	9.6	6.6	0.5
Szamotuły powiat, People's Republic of Poland	52.53	12.8	11.5	9.8	6.5	2.0
Gniezno powiat, People's Republic of Poland	52.55	12.8	11.5	9.8	6.5	2.0
Międzychód powiat, People's Republic of Poland	52.61	12.8	11.5	9.8	6.5	2.0
Oborniki powiat, People's Republic of Poland	52.74	12.8	11.5	9.8	6.5	2.0
Wągrowiec powiat, People's Republic of Poland	52.81	12.8	11.5	9.8	6.5	2.0
Czarnków powiat, People's Republic of Poland	52.94	12.8	11.5	9.8	6.5	2.0
Chodzież powiat, People's Republic of Poland	52.95	12.8	11.5	9.8	6.5	2.0
Trzcianka powiat, People's Republic of Poland	52.95	12.8	11.5	9.8	6.5	2.0
Bydgoszcz, People's Republic of Poland	53.12	12.8	11.5	9.8	6.5	2.0
Szczecin region, Republic of Poland	53.43	12.8	11.5	9.8	6.5	2.0

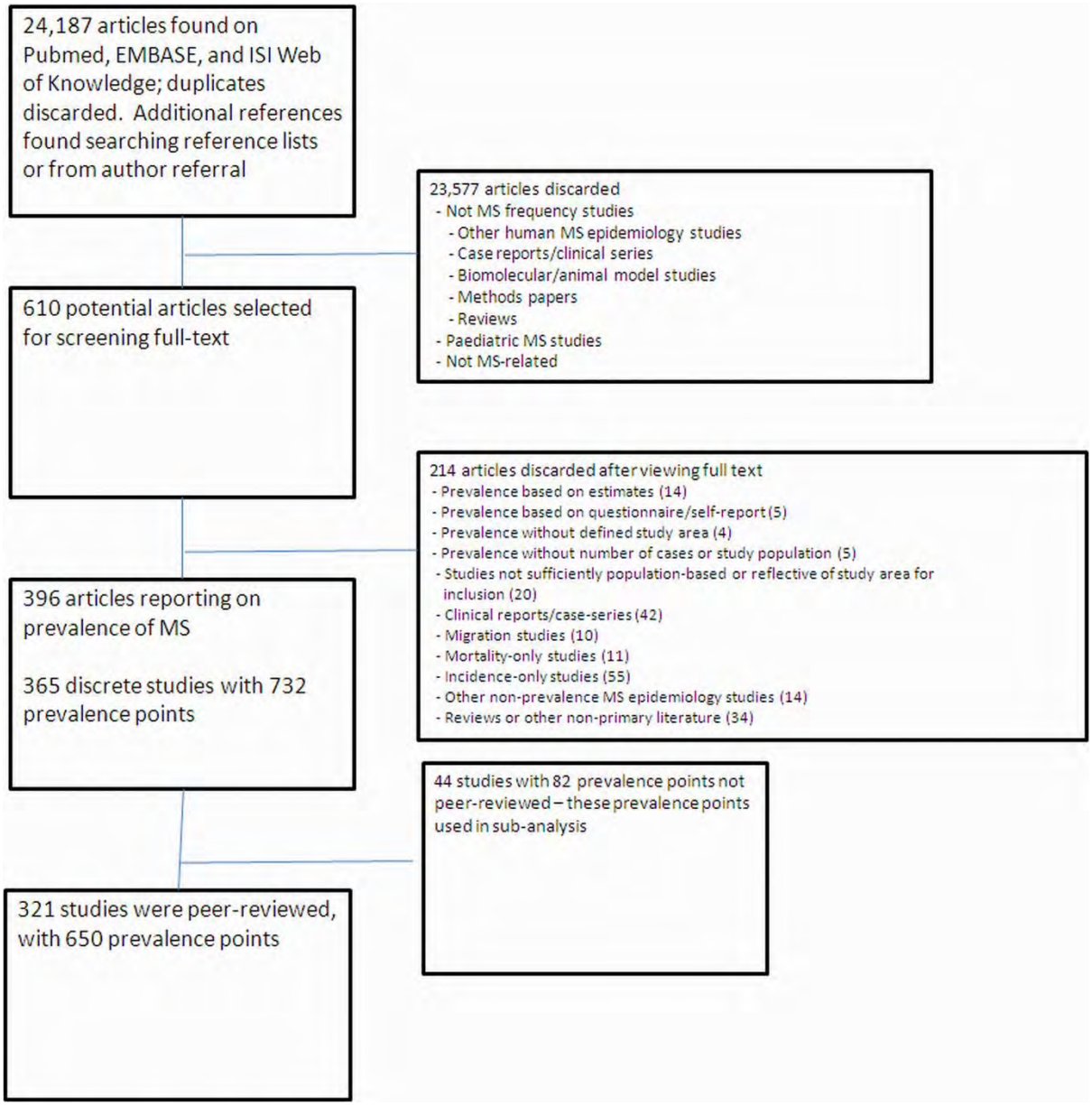
Appendix 3D.6 Middle East & Africa

Republic of Malta	35.94	8.7			21.8	

Note: All place names are those used at the time of the study in question – some may no longer exist or may have been renamed.

Abbreviations:
In UK region/Scandinavia and North Atlantic region: UK=United Kingdom of Great Britain & Northern Ireland.

Appendix 3E. MOOSE criteria flow chart for studies included and excluded from meta-analysis.



Appendix 3F. Publication of "Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis"

Simpson, Jr. SL, Blizzard, L, Otahal P, van der Mei I, Taylor B. "Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis." *Journal of Neurology, Neurosurgery & Psychiatry*. **doi:10.1136/jnnp.2011.240432**.

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Chapter 4. The varied mechanisms of vitamin D in the onset and clinical course of MS: potential roles in modulating other etiological pathways

4.1 Preface

The previous chapter provided strong evidence for a positive association between MS prevalence and increasing latitude. One of the most prominent factors which varies with latitude is winter UVR and vitamin D. Vitamin D has been a major focus of study within MS research, with a range of studies, including cross-sectional and case-control, as well as some cohort studies, demonstrating a strong relationship between increased personal UVR exposure, greater vitamin D intake and circulating metabolites of vitamin D and risk of MS, as well as moderating clinical course.

This chapter will review the biological pathways of vitamin D metabolism and biological function, both intracellular and genomic effects, and its immunomodulatory effects. The epidemiological studies linking UVR and vitamin D with MS frequency and clinical course will be reviewed. Finally, potential roles for vitamin D in modulating or even potentially mediating other recognised pathways in MS aetiology and clinical course, including acute infections, smoking and stress, will be described.

4.2 Introduction

Multiple sclerosis (MS) is a chronic central nervous system (CNS) disorder characterized in the majority of cases by relapsing-remitting inflammatory demyelination on a background of slowly progressing neurodegeneration.(1) The aetiology of MS is complex, with genetic and lifestyle determinants affecting pathogenesis and clinical course. A growing body of work indicates that sunlight and vitamin D (vitamin D) may be involved. While there have been a number of reviews which examine vitamin D and its role in MS with varying emphases, none have yet evaluated the role of vitamin D in mediating or modulating other factors associated with MS risk, particularly acute and latent infection, smoking, pregnancy and stress. Each of these factors has been independently associated with MS; however the mechanisms of these associations are uncertain. As vitamin D is associated with each of these risk factors, vitamin D may have a role in the mode of action of each of these factors, in addition to its independent associations with MS. This chapter was an invited review from Current Medical Literature Neurology (Appendix 4A).

4.3 Background

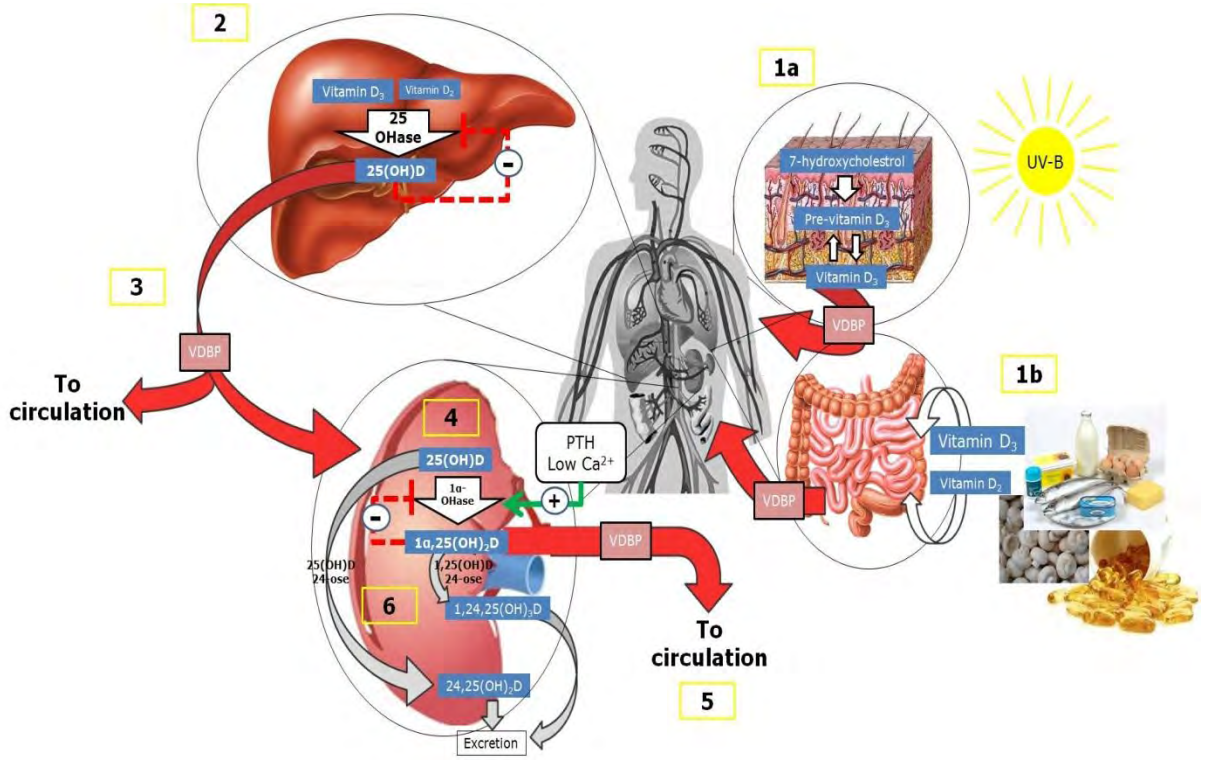
4.3.1 Production and functions of vitamin D

Vitamin D is actually composed of two forms: cholecalciferol (vitamin D_3) is the primary vitamin D precursor, being principally produced endogenously by spontaneous epidermal, cleavage of the β ring of the precursor, 7-dehydro-cholesterol (pre-vitamin D_3), under the influence of solar ultraviolet radiation(2, 3); there are few dietary sources of vitamin D_3; oily fish being the most important naturally occurring source. The second form of vitamin D, ergocalciferol (vitamin D_2), is derived from dietary sources, but is less efficiently absorbed than D_3. Regardless of its form, once in circulation, vitamin D is transported to the liver, where it is hydroxylated to the major circulating form, 25-hydroyvitamin D (25(OH)D). From the liver, 25(OH)D is transported to the kidneys, where it is converted to the active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$).(4) 1,25(OH)2D has a very short half-life

in serum, however, being tightly regulated by the endocrine system's parathyroid hormone to maintain calcium homeostasis, and thus the diagnostic form used to assess vitamin D is 25(OH)D, which has a half-life of 30-days in serum(4) (Figure 4.1).

Figure 4.1. Vitamin D metabolism

Vitamin D3 is produced via two pathways – 1a) 7-hydroxycholestrol is converted to pre-vitamin D3 via photolysis from UVRB, thereafter spontaneously isomerising to vitamin D3. 1b) Alternatively vitamin D3 (cholecalciferol) may be absorbed in the intestines from certain foods, including dairy products, eggs and principally fatty fish and also in dietary supplements where it is made by UVRB irradiation of animal fats; vitamin D2 (ergocalciferol) is solely found in dietary supplements, and is made by UVRB-irradiated fungi and brewer's yeast. After absorption both types of vitamin D are bound to the vitamin D binding protein (VDBP) and transported to the liver. 2) In the liver, vitamin D is hydroxylated to 25-hydroxyvitamin D 25(OH)D by the enzyme vitamin D 25-hydroxylase, regulated by negative feedback from 25(OH)D. 25(OH)D may then be stored in hepatocytes until needed. 3) From the liver, 25(OH)D is transported, largely bound to VDBP, either to the kidneys or to other cells. 4) In the kidneys, 25(OH)D is converted to 1,25(OH)2D via the enzyme 25-hydroxyvitamin D 1-alpha-hydroxylase, a process which is upregulated by parathyroid hormone (PTH) and low serum calcium (Ca2+), as well as negative feedback from 1,25(OH)2D and is mainly involved in classical calcium homeostasis. 5) Additionally 1,25(OH)2D is transported to the general circulation, largely bound to VDBP. 6) Both 25(OH)D and 1,25(OH)2D may also be degraded in the kidneys, to 24,25(OH)2D and 1,24,25(OH)3D, and thence to calcitriolic acid and excreted in the urine. Additional non endocrine conversion of 25(OH)D to 1,25(OH)2D can occur in many other cell types (see text).



While the majority of circulating $1,25(OH)_2D$ is produced in the kidneys, there is local conversion of 25(OH)D to $1,25(OH)_2D$ in a variety of cell and tissue types.(5) In contrast to the endocrine production of $1,25(OH)_2D$, local production appears to be more dependent on the serum level of

25(OH)D and the local cytokine milieu.(6) Of especial relevance for its role in MS, this conversion takes place in many cell types of the immune system(7), as well as in the neuronal and glial cells of the CNS.(5, 8).

$1,25(OH)_2D$ has been demonstrated *in-vitro* to modulate the immune response(9), depressing pro-inflammatory immune cell activity, while stimulating T_h2 and T_{reg} activity. $1,25(OH)_2D$ depresses or inhibits the production of T_h1 cytokines(7) and the T_h17 cytokine IL-17,(10) while stimulating the production of T_h2 and T_{reg} cytokines.(7) In the CNS, $1,25(OH)_2D$ has been found to block the production of pro-inflammatory cytokines and nitric oxide by microglia.(11) Treatment of astrocytes with $1,25(OH)_2D$ makes them less responsive to stimulation with pro-inflammatory signals.(12)

Additionally, $1,25(OH)_2D$ is necessary for neurotransmitter and neuronal function.(13) Indeed, maternal UVR exposure and levels of 25(OH)D have been found to have a significant role on the brain development of offspring in murine models(14). Recently, a fascinating role for $1,25(OH)_2D$ in moderating demyelination and potentiating remyelination was demonstrated,(15) preventing apoptosis of oligodendrocytes and promoting the health and function of existing cells, and stimulating the differentiation of precursors into functional oligodendrocytes

4.3.2 Cellular & genetic activity of $1,25(OH)_2D$

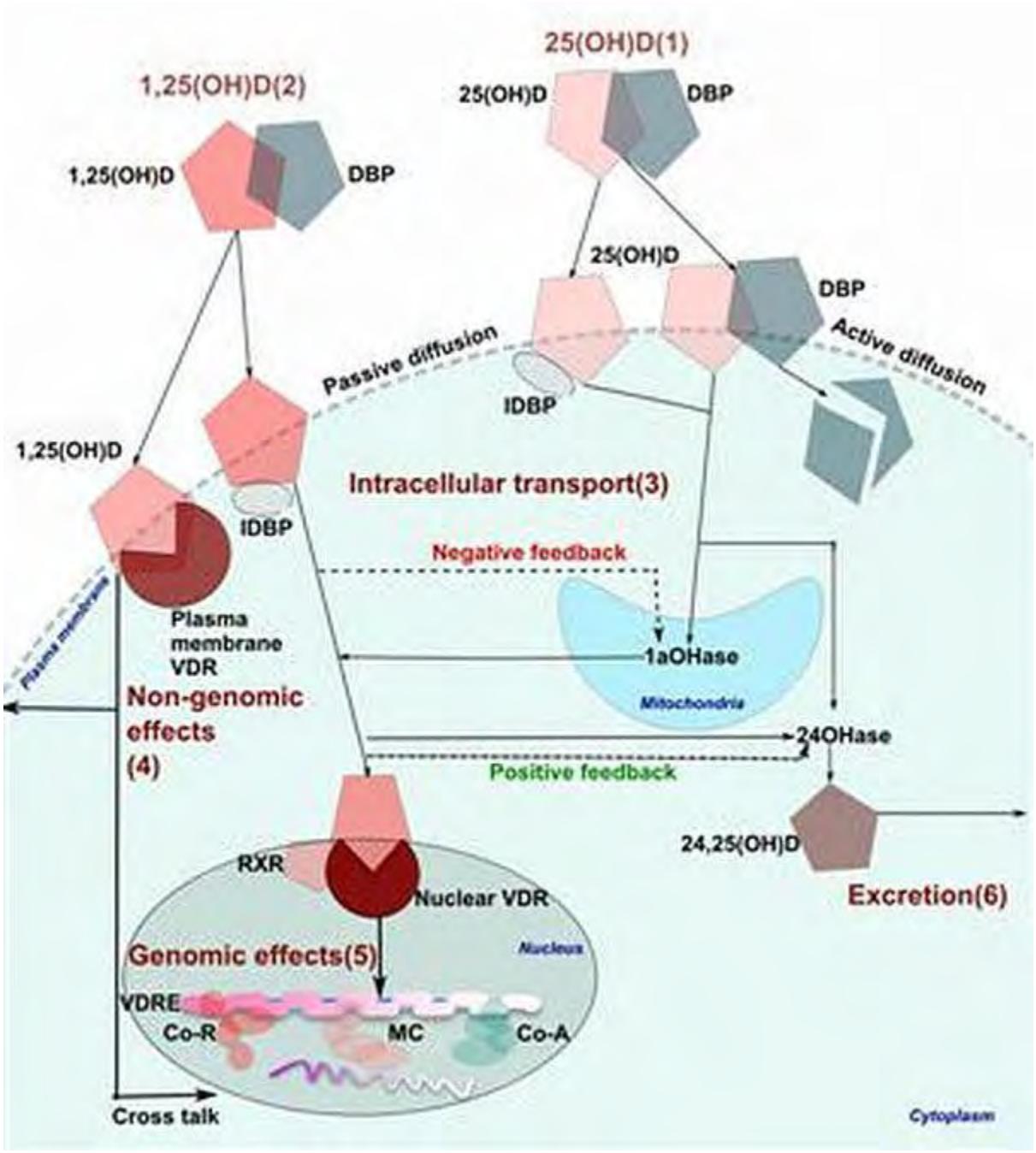
Vitamin D can act upon target cells via two pathways: as 25(OH)D bound to the vitamin D transport protein, where it is converted to $1,25(OH)_2D$ in the mitochondria of capable cells and thence bound to vitamin D receptor (VDR) to exert its genomic effects(16); or as free $1,25(OH)_2D$, produced in the kidneys (endocrine), in other local cells (paracrine) or from the cell itself (autocrine), can bind to or cross the cell membrane and exert rapid and intermediate, non-genomic effects (Figure 4.2).

The rapid effects of $1,25(OH)_2D$ begin within seconds of exposure(16, 17), acting principally at the level of the plasma membrane, affecting ion channels and membrane-bound signal-transduction. Additionally, $1,25(OH)_2D$ has various intermediate effects(16, 17), occurring hours after initial

exposure, including modulating protein kinases. Some of these rapid effects of $1,25(OH)_2D$ have been found to act independently of VDR, allowing the possibility of vitamin D activity in a broader range of cell types than presently recognised(18). These rapid and intermediate effects, particularly the phosphorylative effects of activated protein kinases, act to modulate the genomic effects of $1,25(OH)_2D$ which occur much later.(16, 19)

Figure 4.2. Pathway of $1,25(OH)_2D$ in cell and gene regulation.

1) 25(OH)D and 1,25(OH)D are predominantly transported in plasma bound to vitamin D binding protein (VDBP). A small proportion, 0.04% of 25(OH)D and 0.4% of 1,25(OH)D are transported unbound in the plasma and has traditionally been assumed to be the fraction that enters target cells via passive diffusion. DBP bound 25(OH)D is now recognised to be internalised via receptor mediated membrane transport. 2) Once in the cell 25(OH)D is transported to the mitochondria where it undergoes its second hydroxylation to 1,25(OH)D by the enzyme 1α-hydroxylase (1aOHase) encoded by the gene *CYP 27B1*. 1,25(OH)D also directly enters the cell. The level of intracellular 1,25(OH)D is controlled by a feedback system. 1,25(OH)D reduces the expression of *CYP 27B1* thereby reducing the conversion of 25(OH)D to 1,25(OH)D and induces expression of *CYP24A1* the gene encoding the enzyme 24-hydroxylase (24OHase). 3) The movement of 25(OH)D and 1,25(OH)D from the cell membrane to its intracellular destinations, is a highly ordered process involving a chain of intracellular vitamin D binding proteins (IDBPs). IDBPs have recently been recognised to play an important role in the modulation of the genetic effects of $1,25(OH)_2D_3$ and have been shown to enhance the effect of VDR-mediated signalling and to have an effect on *CYP27B1* 4) Non-genomic effects occur on binding of 1,25(OH)D to the cell membrane VDR and take place within the plasma membrane and cytoplasm of the cell. The rapid effects, occur within seconds to minutes of $1,25(OH)_2D$ binding include the modulation of ion or ligand-gated ion channels and the production of cAMP or IP3 at the cell membrane. The intermediate effects occur hours of 1,25(OH)D binding and include the phosphorylation or dephosphorylation of protein kinases and other enzymes, thereby modulating genetic expression and cell function 5) Genomic effects of 1,25(OH)D primarily occur within the cell nucleus. The vitamin D receptor (VDR) forms a heterodimer with retinoid X receptor (RXR) and this complex preferentially binds 1,25(OH)D. The resulting complex binds vitamin D response elements (VDRE) on target genes, resulting in dissociation of co-repressors (CoR) and recruitment of co-activators (CoA) . The VDR-CoA interaction facilitates recruitment of mediator complexes (MC) that build a bridge between the VDRE the transcription machinery to affect expression of genes. These genomic responses modulate the production of new proteins over hours to days. 6) 25(OH) D and 1,25(OH) D are catabolised by the enzyme 24-hydroxylase (24OHase) to the inactive, water soluble form calcitroic acid (24,25(OH)) for renal excretion



Vitamin D exerts its genomic effects via the transport of 25(OH)D from the vitamin D binding protein, conversion to $1,25(OH)_2D$ in the mitochondria, and interaction with VDR, in a highly ordered process involving a chain of chaperones and co-chaperones (Figure 5.2).(16) The vitamin D-VDR complex binds to the vitamin D response element (VDRE), up-regulating or repressing transcription(16). Some of these genetic effects act to regulate vitamin D metabolism(20, 21), but also affect a range of genes

related to cellular proliferation/differentiation(22, 23), and immune-relevant genes, including cytokines and cytokine receptors.(23, 24). Also, Ramagopalan and colleagues(25) recently demonstrated that the strongest MS susceptibility region, the HLA-DRB1*1501 allele of the HLA-DR gene, is up-regulated by $1,25(OH)_2D$ via a VDRE.

Not unexpectedly, polymorphisms in the genes encoding the key proteins in the actions of vitamin D, including VDR (*VDR*) and 1α-hydroxylase (*CYP27B1*), have been associated with MS risk. Polymorphisms in *CYP27B1* have been associated with significantly enhanced risk of MS;(26) however in other studies, no significant associations were found (27, 28). The evidence for an association between polymorphisms in *VDR* is mixed, with some finding an association(29-31), but others not(27, 28, 32-34) . Also, interactions between *VDR* polymorphisms and HLA-DR15(35), sun exposure(32) or dietary vitamin D intake(28)have been observed, but these have not yet been confirmed.

4.4 Epidemiology

Much of the evidence relating vitamin D and UVR exposure to MS aetiology has been derived from epidemiological studies which found significant correlations between MS prevalence and incidence rates to latitude, season, month of birth, migration and UVR exposure.

4.4.1 Latitude, season, UVR and MS

It is generally recognised that there is a distinct latitudinal gradient in the distribution of MS frequency – prevalence and incidence surveys found increased frequencies at higher latitudes.(36, 37) Some have suggested that the distribution is a manifestation of genetic susceptibility and migration(38), but this does not account for the gradients within nations, including relatively genetically-homogenous nations like Australia and New Zealand.(39, 40) Also, migration between areas of different MS risk below a critical age connotes the risk of MS of the new residence, whereas migration after this age connotes the risk of the original residence.(41)

Summer season of gestation has been significantly associated with MS aetiology and clinical course. A number of studies, including large, pooled-cohort studies(42) and a sibling case-control study with nearly 11,500 sibling pairs,(43) found that gestation during summer months, with birth during autumn/early winter is significantly protective against later development of MS. Among persons with MS, gestation during summer affects subsequent disease, with those not born in summer having an earlier onset of disease.(44) This appears to be a manifestation of UVR exposure: a recent study found that the association between season of birth and MS was abrogated after adjustment for region of birth and the ambient UVR during the mother's first-trimester,(45) suggesting that the effects of season of birth are due to maternal UVR exposure.

There is also a significant seasonality of disease activity, with prospective cohort studies finding more clinical and MRI-detected exacerbations during winter.(46, 47) This effect too may be due to the change in ambient and personal UVR with winter season at higher latitude correlating with reduced ambient UVR, as well as reduced time outdoors and increased clothing.(48).

There is substantial additional support from studies that measured past UVR exposure. Higher sun exposure in childhood or adolescence has been associated with a reduced risk of MS, in a study of disease-discordant monozygotic twins,(49) as well as in case-control studies in Tasmania(50) and Norway(51). Occupational sun exposure has been associated with reduced MS mortality(52), and in a record linkage study(53), where skin cancer was used as a surrogate measure of personal sun exposure, skin cancer was significantly less common in people with MS than in the comparison cohort. Also, increased ambient UVR exposure shows a significant inverse relationship with exacerbation rates.(47, 54)

4.4.2 Vitamin D and MS

4.4.2.1 Vitamin D and MS aetiology

Dietary intake of vitamin D has not shown any significant effect on subsequent risk of MS. A large prospective pooled-cohort study of 238,371 women, combining data from the two Nurses Health Studies, found no effect of dietary sources of vitamin D on subsequent risk of MS. However, this study did find a significant and dose-dependent inverse relationship between vitamin D supplement intake at baseline and subsequent risk of MS; a similar but weaker association was also found for cumulative supplement intake.(55) A subsequent study assessing diet and supplementation during adolescence(56) found no significant effect from either, suggesting that the time of action for vitamin D intake may operate outside adolescence.

While vitamin D intake has not been associated with MS risk, this may be due to some extent to the subjective and imprecise measurement of intake behaviour, as well as the minor source that diet is for vitamin D in most European populations.(57) A more accurate method of assessing vitamin D is by measuring the levels of circulating metabolites. There have been a number of studies which examine the relationship of serum levels of vitamin D metabolites, either 25(OH)D or $1,25(OH)_2D$, with MS risk, the majority of which show significantly lower levels in cases versus controls. Additionally, the one study which evaluated vitamin D prospectively as a predictor of MS risk, by Munger and colleagues, report the odds of MS are reduced by over a third per 50nmol/L increase in serum 25(OH)D, measured years before onset; however this could only be demonstrated among whites, as no African-American subjects had 25(OH)D levels above 99nmol/L, where a significant inverse association was demonstrated.(58) It may be that circulating levels of 25(OH)D infrequently reach the concentrations needed to exert a significant protective effect against MS in persons of African descent, given the high melanin content in the skin hinders vitamin D synthesis in the skin. However given small number of

African-American subjects in this study (77 cases, 154 controls), relative to that of whites (148 cases, 296 controls), these findings may simply be an artifact of sample size. Of interest is that among Hispanic individuals, in whom melanin content is between that of African-Americans and whites, 25(OH)D levels above 99nmol/L were found and a protective effect observed for higher 25(OH)D; however due to the small number of Hispanic participants (32 cases, 64 controls), these results were not statistically significant ($p=0.54$). Correale and colleagues found significantly higher 25(OH)D levels between controls and cases of RRMS, during RRMS ($p<0.00001$) and even more so for exacerbation samples ($p<0.0001$); similarly for 1,25(OH)2D, levels were significantly higher in controls relative to RRMS remission samples ($p<0.0003$) and more so for exacerbation samples ($p<0.0001$).(59) For PPMS, however, levels of 25(OH)D ($p=0.70$) and 1,25(OH)2D ($p=0.30$) were not significantly different between cases and controls. These findings are unlikely to be due to sample size, as the number of RRMS ($n=58$) and PPMS ($n=40$) were similar. These studies by Munger(58) and Correale(59) suggest there may be some differences in the effects of vitamin D by race and MS course; however further studies with larger samples sizes are needed to evaluate this further.

Overall, this data provides strong evidence that vitamin D and/or exposure to UVR are causally related to MS onset.

4.4.2.2 Vitamin D and clinical course

Studies examining the role of vitamin D in MS clinical course are almost uniform in their demonstration of an inverse association between serum vitamin D and clinical exacerbation. The majority of such studies do so in a cross-sectional fashion, comparing the levels of serum 25(OH)D and $1,25(OH)_2D$ between exacerbation and remission samples, therefore precluding ascription of causality. However, two cohort studies have provided evidence in favour of a causal association. Simpson, Jr. and colleagues prospectively followed a cohort of 145 RRMS patients for a mean of 2.3 years, finding a reduction in the hazard of exacerbation of 12% per 10nmol/L increase in serum 25(OH)D.(60) Similarly Mowry

and colleagues found a 44% reduction in the risk of exacerbation per 25nmol/L increase in serum 25(OH)D in a retrospective cohort of 110 paediatric-onset MS cases.(61)

While serum vitamin D metabolites have shown strong evidence of an inverse relationship with clinical exacerbation, no studies have yet demonstrated any significant association between vitamin D metabolites and MRI activity. Also only one study(62) to-date has evaluated CSF levels with MS clinical course, finding no association.

A number of cross-sectional studies have evaluated serum 25(OH)D and disability progression, finding significant inverse associations between vitamin D and increased disability. Of note is the retrospective cohort study by Smolders and colleagues, which found a significant inverse association between serum 25(OH)D and EDSS,(63) and the recent study by Weinstock-Guttman and colleagues, which found a significant inverse association between baseline-measured serum 25(OH)D and MSSS.(64) However, the single-measure nature of these studies precludes any establishment of a causal relationship. Indeed, there is a strong case to be made that these findings are due to reverse causality, given the relationship between reduced mobility due to disability and consequent reduced UVR exposure and vitamin D. Also, Uhthoff's phenomenon need be taken into consideration in evaluating the role of vitamin D in progression – if persons with more active disease avoid sun so as to prevent the exacerbation of disease by the heat, they will consequently have lower vitamin D, resulting in a spurious link with progression. Prospective cohort studies utilising serially-measured vitamin D and personal sun exposure behaviours, and longitudinally-measured progression are needed to tease out the nature of this association.

There has been some evidence of a role for vitamin D supplementation in moderating MS clinical course, including the recent prospective randomised-controlled trial (RCT) by Burton and colleagues,(65) which found significantly reduced annual exacerbation rates and a moderated increase in EDSS amongst those randomised to a graded vitamin D supplementation regimen up to 280,000IU/week over 12-months, relative to supplements up to 4000IU/day. This study prevents

definitive conclusions because it was designed to assess tolerability of the high dose schedule, had a small sample size (n=25 treatment, 24 placebo) and there was a difference in the clinical visit frequency between the two arms. However these results are encouraging and warrant further investigation with larger cohorts.

4.5 Vitamin D and other aetiologic factors

Much interest has centered on the direct effect of vitamin D on MS onset and clinical course via its immunomodulatory and genomic effects described earlier. However it is also possible that vitamin D may act as a confounder for other factors shown to play a role in MS aetiology or clinical course. Additionally, these factors may exert independent effects which are modulated by vitamin D, having stronger or weaker effects depending on the level of vitamin D. Below are a number of hypothetical modes of action by vitamin D which might be investigated.

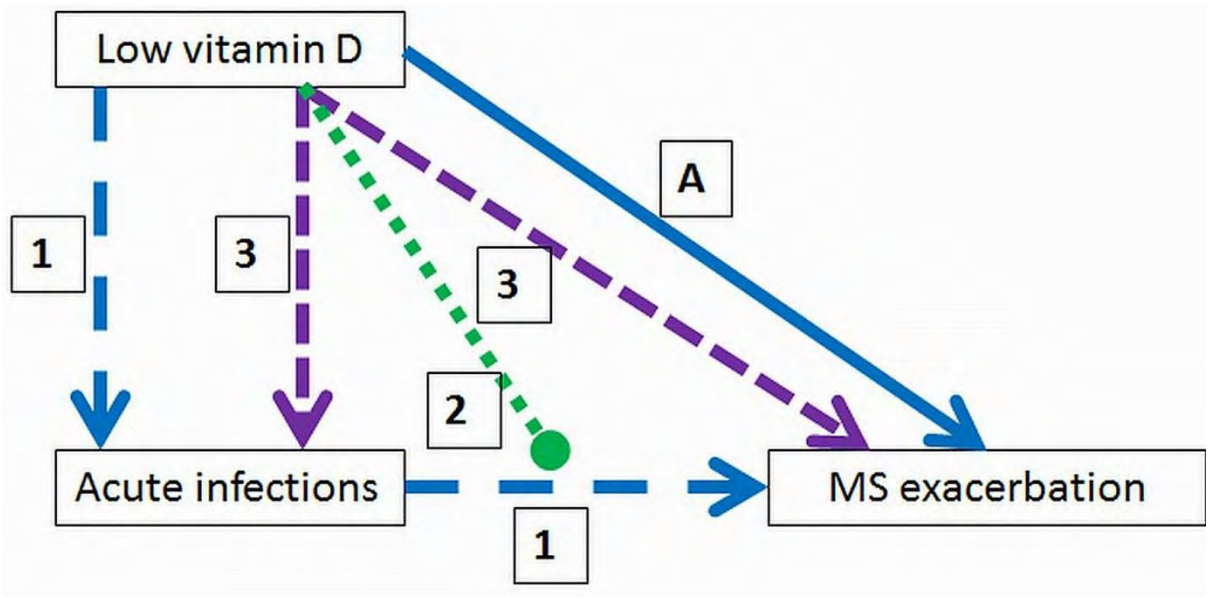
4.5.1 Vitamin D and acute infections

In adulthood, acute infections,(66) principally upper respiratory tract infections (RTI), have been associated with a significantly greater risk of exacerbation. However, a causal link between RTI infections and MS exacerbations has not been demonstrated. At the same time, lower levels of serum 25(OH)D have been associated with acute infections.(67) Recently, an RCT of daily vitamin D supplementation (400IU/day) found a trend toward a protective effect against acute infections.(68)

As in Figure 4.3, low vitamin D levels could increase susceptibility to acute infections which, in turn, independently increase the risk of exacerbations (Pathway 1). It could also be that the effect of acute infections on exacerbation risk is enhanced by lower levels of vitamin D, with an increased pro-inflammatory immune response which could contribute to neuropathology (Pathway 2). Another possibility is that the association between acute infections and MS exacerbations is not reflective of an aetiologic relationship but rather, a common outcome of lower levels of vitamin D manifesting in both infection and MS exacerbation (Pathway 3).

Figure 4.3. Vitamin D & acute infections.

In addition to its own independent effects on the risk of MS exacerbation (A), low vitamin D could increase the risk of acute infections, which then independently increase the risk of exacerbation (Pathway 1). Additionally, vitamin D could modulate the effects of an independent effect of acute infection on risk of MS exacerbation (Pathway 2). Also, it may be that the association found between acute infection and exacerbation is an artefact, a consequence of both acute infection and MS exacerbation being shared outcomes of low vitamin D (Pathway 3).



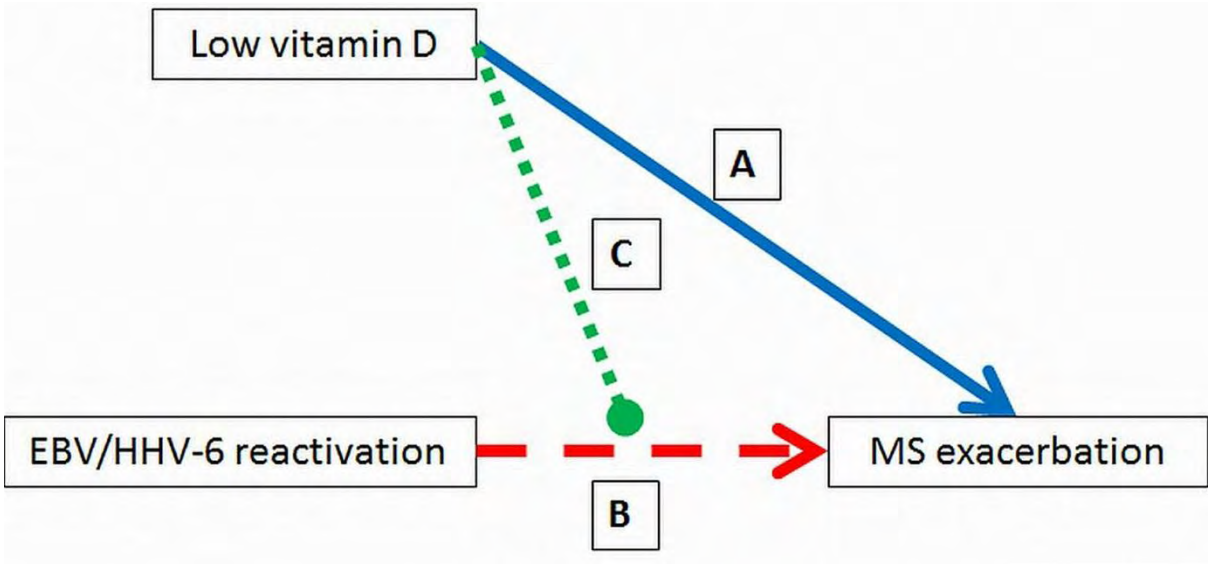
4.5.2 Vitamin D and herpesvirus infection

Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV-6) have been associated with MS risk(69, 70) and clinical course.(71, 72) Also, interactions have been found between EBV and HHV-6 and vitamin D in mediating MS, most notably the recent finding that the EBNA-3 product of EBV directly blocks the action of VDR in activating its target genes.(73)

Hypotheses have been proposed whereby EBV may interact with vitamin D to manifest in MS. Holmøy(74) suggests that lower levels of available vitamin D might coincide with reactivation of EBV (Figure 4.4).

Figure 4.4. Vitamin D & herpesvirus reactivation: Holmøy hypothesis.

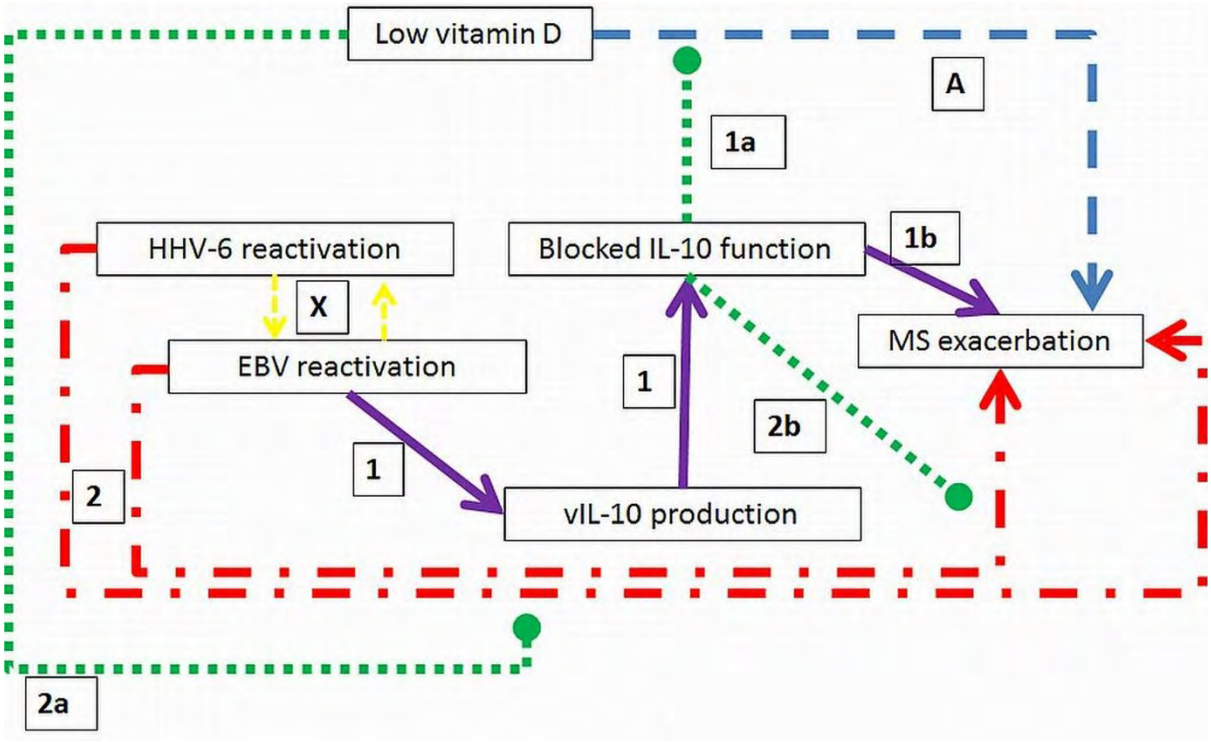
The Holmøy hypothesis suggests that, in addition to its own immunomodulatory effects (Pathway A), low vitamin D may co-occur with reactivation of latent EBV and/or HHV-6 infection, enhancing (Pathway C) their independent pathogenic effects (Pathway B).



Hayes and colleagues(75) propose a novel interaction between lower levels of vitamin D and EBV via the IL-10 analogue vIL-10 produced by EBV, which binds to the IL-10 receptors, interfering with T_h2, anti-inflammatory functions of IL-10. These effects of vIL-10, combined with the absence of the immunomodulatory functions of vitamin D could manifest in a more inflammatory state, predisposing to MS activity (Figure 4.5). These effects would proceed alongside the effects of the viral replication and resultant immune response, as in the Holmøy hypothesis, which could in turn by modulated by low vitamin D levels.

Figure 4.5. Vitamin D & herpesvirus reactivation: consolidated

The Hayes hypothesis suggests that production of the EBV viral analogue of the Th2 cytokine IL-10, in interfering with proper function of the human IL-10, may lead to a pro-inflammatory state, predisposing MS exacerbation (Pathway 1). The low vitamin D levels, in addition to their independent effects (Pathway A) would synergise with the effects of vIL-10 (Pathway 1a). At the same time, the antigenic effects of EBV/HHV-6 reactivation could lead to pathogenic effects, these intensified both by low vitamin D (Pathway 2a) and by the immunomodulatory effects of vIL-10 (Pathway 2b). Of note, the capacity of EBV and HHV-6 to transactivate both partial and full transcription of one another's latent genomes allows the possibility for reactivation of HHV-6 to activate the EBV vIL-10 pathways (Pathway X)



Of note, since HHV-6 and EBV have the ability to trans-activate latent infections of one another,(76) HHV-6 could play a role in the hypothesised pathways of Holmøy(74) and Hayes.(75)

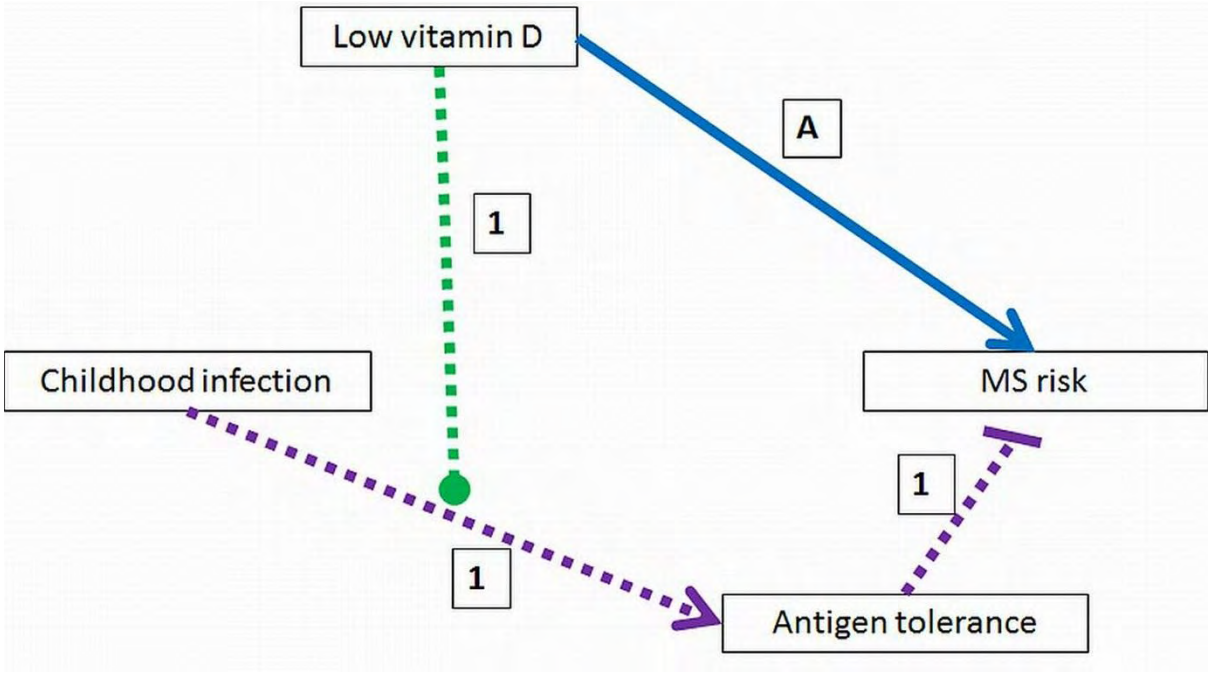
4.5.3 Vitamin D and childhood infections

A greater burden of, and earlier exposure to, common childhood infections has been associated with a reduced risk of subsequent MS. This relationship may potentially be related to vitamin D. Ponsonby and colleagues(77) found the protective effect of greater infant sibling exposure was largest in persons with higher winter UVR exposure, with those children with <1hour/day winter exposure having no significant protective effect from increased sibling exposure. Also, lower levels of serum 25(OH)D

have been associated with acute infections in children.(78) It may be that the tolerogenic effect of childhood exposure to pathogens is only effective for those whose vitamin D is above a threshold level and below this level otherwise tolerogenic exposures are instead pathogenic (Figure 4.6).

Figure 4.6. Vitamin D & childhood infections

In addition to the pro-inflammatory state left by low vitamin D, which may have independent effects leading to MS (Pathway A), sufficient levels of vitamin D in critical periods of childhood immune tolerance training may be required for childhood infectious exposure to lead to immune tolerance (Pathway 1). In the absence of the immunomodulation of vitamin D, these antigenic exposures may instead lead to a pro-inflammatory immune response against antigens with sufficient similarity to self-antigens to lead to autoimmune reactions as found in MS.



4.5.4 Vitamin D and stress

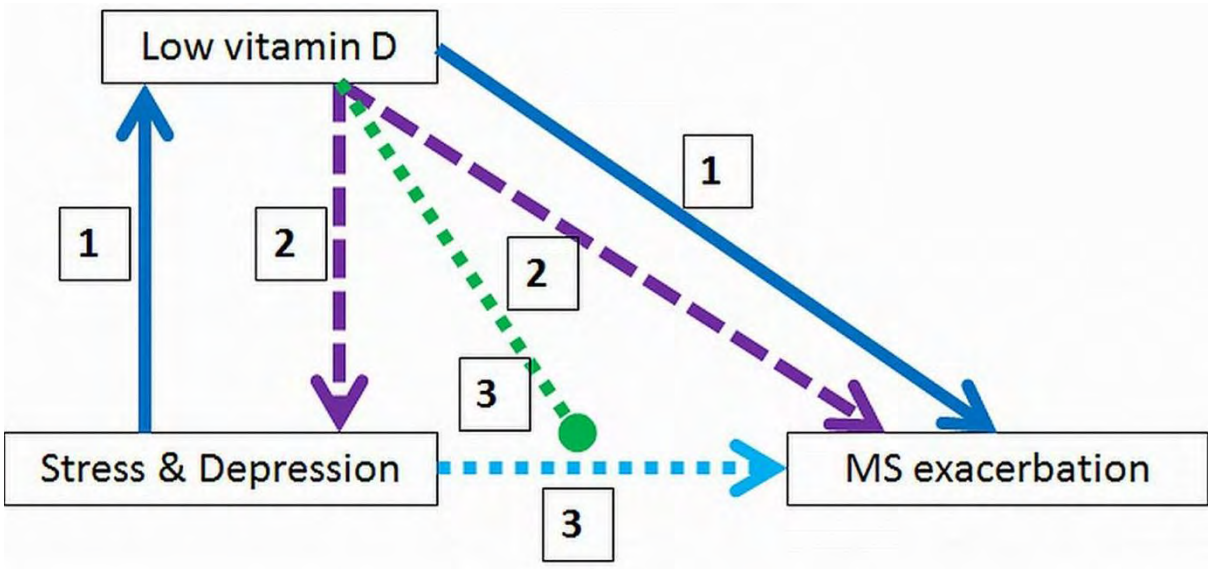
Stress has been associated with clinical course in MS, with a significantly greater risk of exacerbation after stressful life events.(79) The mechanism by which stress may manifest in exacerbation of MS is as complex as stress itself; however it is increasingly recognised that there is a powerful interaction between emotional state and the innate and adaptive immune system.(80) Also, human(81) and animal(82) models have found significantly altered immunological parameters in those with stress and depression. At the same time, vitamin D has been associated with depression, with lower levels of

serum 25(OH)D(83) in persons with clinical depression(83, 84). Reverse causality is a potential concern; however, an RCT wherein subjects with depression (n=441) were randomized to 20,000 or 40,000 IU/week vitamin D versus placebo for one year found a significant decrease in the depression score.(85).

It may be that stress and depression leads to less vitamin D due to behavioural changes, which could contribute to the relationship between stress and depression and exacerbation (Figure 4.7, Pathway 1). Another possibility is that stress and depression may be a common outcome with MS exacerbation during periods of low 25(OH)D, and the stress-exacerbation association a result of confounding by vitamin D (Pathway 2).

Figure 4.7. Vitamin D & stress

Stress and depression can affect behaviour and energy, resulting in less time spent outdoors and thus, less vitamin D, with resultant pro-inflammatory state due to this vitamin D deficiency (Pathway 1). Another possibility is that stress and depression arise from a shortfall of vitamin D, and the association between stress and depression and MS exacerbation is instead a result of confounding by low vitamin D (Pathway 2). Also, it may be that stress and depression act to manifest in MS exacerbation by some other pathway, which might be enhanced by the pro-inflammatory state caused by insufficient vitamin D (Pathway 3).



These relationships presuppose interchangeability between stress and depression. While both are necessarily related, stress following stressful life events may not lead to frank depression. There is a dearth of literature evaluating vitamin D following transient, non-depressive stress in human models,

however, and such studies should be done. A further possibility is that low levels of vitamin D may, by its contribution to a pro-inflammatory immune profile, enhance independent effects of stress or depression on risk of exacerbation (Figure 4.7, Pathway 3).

4.5.5 Vitamin D and pregnancy

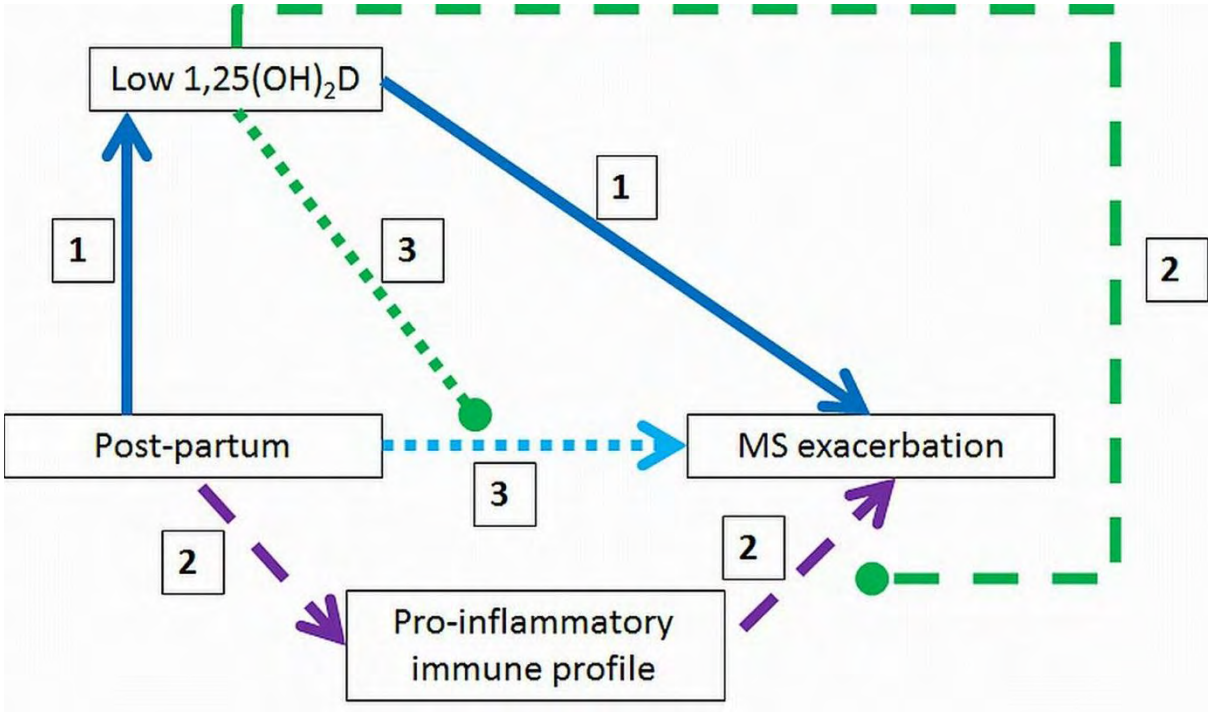
It has long been recognised that the risk of acute exacerbation is significantly reduced during pregnancy,(86, 87) while in the post-partum period, the risk is significantly heightened. The cause of this change in risk profile is unclear. A recent meta-analysis(88) of 20 studies of women at or near term versus 14 studies of non-pregnant women, found that while the circulating levels of 25(OH)D were not significantly different between the two groups, the level of $1,25(OH)_2D$ in pregnant women was nearly double that of non-pregnant females, and the ratio of $1,25(OH)_2D/25(OH)D$ was 2.5-times higher in pregnant vs. non-pregnant females. Also, a study evaluated the cytokines produced by stimulated PBMCs taken from women during and after their pregnancy, found that PBMCs collected during pregnancy produced a T_h2 response, whereas those taken post-partum, showed a markedly T_h1 response.(89)

As it has been demonstrated that the placenta is a site of conversion of 25(OH)D to $1,25(OH)_2D$(90), the higher levels of circulating 1,25(OH)2D may act to induce an anti-inflammatory immune profile, manifesting in a general tolerogenic state, to allow immune tolerance of the 'foreign' offspring. At delivery then, the sudden loss of the higher 1,25(OH)2D produced by the placenta would require an adjustment period for the body, during which time the immune system may have a more pro-inflammatory profile. (Figure 4.8, Pathway 1). It could also be that the change in immune profile acts independently of changes in $1,25(OH)_2D$; however lower levels of $1,25(OH)_2D$ would interact with a heightened pro-inflammatory immune state following pregnancy, increasing the risk of exacerbation (Pathway 2). It may also be that pregnancy manifests in MS exacerbation outside the changes in

immune inflammatory state, in which case the effects of the lower $1,25(OH)_2D$ could modulate this (Pathway 3).

Figure 4.8. Vitamin D & pregnancy

The risk of MS exacerbation in the post-partum period may be due to the sharp fall-off in 1,25(OH)2D, which could manifest in exacerbation due to the resultant pro-inflammatory state (Pathway 1). Another possibility is that the pro-inflammatory Th1 state which arises after pregnancy may occur via some non-vitamin D pathway, which could in turn synergise with the effects of low vitamin D to manifest in exacerbation (Pathway 2). Also, the link between pregnancy and MS exacerbation may be by some other, unknown pathway beyond changes in the Th1/Th2 balance, which could be enhanced by the pro-inflammatory state resultant from low vitamin D (Pathway 3).



In a recent publication, Langer-Gould and colleagues followed 28 pregnant women over their third trimester and 6-months after, finding that, while the risk of exacerbation was significantly higher in the post-partum period, this was associated with higher 25(OH)D in the post-partum period(91). The authors speculate that pregnancy might somehow alter the immunomodulatory effects of vitamin D, instead making it pro-inflammatory and thus, increasing the risk of exacerbation. However, another interpretation of these seemingly paradoxical findings is that they may provide some evidence in favour of Pathway 1. The adjustment period after the loss of the placenta after delivery would likely find an

increase in the levels of circulating 25(OH)D. It may be that in those women with higher 25(OH)D in the post-partum period in the Langer-Gould cohort have lower conversion rates of 25(OH)D to 1,25(OH)2D relative to those women with static 25(OH)D after delivery. Thus, after the loss of the placenta, those women with static 25(OH)D had had less of a decline in their production of 1,25(OH)2D and less risk of exacerbation, whereas in the other group, the conversion capacity in the absence of the placenta was less, resulting in a greater loss of circulating 1,25(OH)2D and greater risk of exacerbation. Studies similar to that of Langer-Gould and colleagues should be done, but in which 1,25(OH)2D is measures over time along with 25(OH)D, to evaluate this possibility.

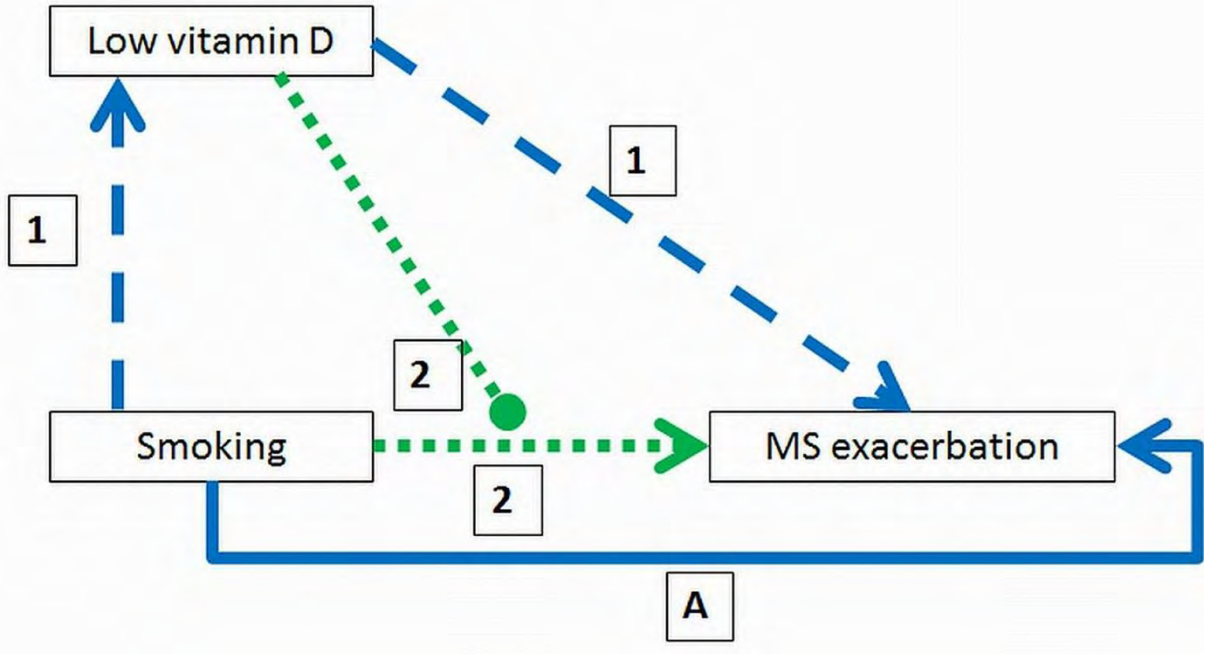
4.5.6 Vitamin D and smoking

Tobacco smoking and passive exposure have been associated with MS aetiology(92) and clinical course.(93, 94) At the same time, smoking has a known detrimental effect on vitamin D absorption and metabolism,(95) with levels of serum vitamin D metabolites significantly lower in smokers relative to non-smokers.

It may be that the effect of smoking on vitamin D metabolism could be a component of its relationship with MS, with smoking and passive smoke exposure contributing to lower levels of serum 25(OH)D and 1,25$(OH)_2$D, and thence, affecting MS risk and course due to an increased pro-inflammatory immune state (Figure 4.9, Pathway 1). At the same time, lower levels of vitamin D could enhance independent effects of smoke exposure on the risk of exacerbation (Pathway 2), with the pro-inflammatory state resultant from the absence of vitamin D immunomodulation intensifying the effects of smoking.

Figure 4.9. Vitamin D & smoking

Smoking negatively affects vitamin D absorption and metabolism, which in so reducing levels of vitamin D, could lead to a pro-inflammatory state, leading to MS exacerbation (Pathway 1); these effects on vitamin D would occur alongside the other vitamin D independent effects of smoking which lead to MS exacerbation (Pathway A). Another possibility is that low vitamin D might modulate the effects of cigarette smoking, either via the pro-inflammatory state resultant from low vitamin D, or in some other fashion (Pathway 2).



4.6 Conclusion

The evidence is accumulating that vitamin D is causally related to MS onset and clinical course. Increasing knowledge shows the high complexity of the biological pathways by which vitamin D exerts its effects on the immune system and CNS. There is also the possibility that vitamin D is affecting or even mediating the relationships between several leading factors and MS aetiology or clinical course, including acute infection, smoking, pregnancy and stress. Therefore, well-designed cohort studies should measure all key factors and examine these possibilities. In relation to treatment of MS patients, randomised controlled trials of vitamin D therapy are now needed to establish whether vitamin D treatment can reduce exacerbations and/or disease progression and which dose is most appropriate.

4.7 Summary

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system. The aetiologies of MS onset and clinical course are complex and have not yet been fully explained; however a leading environmental candidate is vitamin D. The well-recognised immunomodulatory effects of vitamin D, inducing an anti-inflammatory and tolerogenic immune profile, along with the virtual ubiquity of vitamin D receptors and conversion enzymes in the cells of the immune system and central nervous system, gives vitamin D a strong biological plausibility for this role. This biological evidence is supported by a wealth of epidemiological evidence, both indirectly via latitudinal gradients in MS frequency and directly by inverse associations between MS risk and clinical course and personal ultraviolet radiation exposure and circulating levels of vitamin D. Beyond the immunomodulatory mechanisms by which vitamin D affects MS, there may be a role for vitamin D in modulating or even mediating the pathways of other, independently demonstrated predictors of MS onset and clinical course, including acute infection, childhood infection, herpesvirus reactivation, pregnancy, stress and smoking. Prospective, randomised clinical trials of vitamin D are strongly indicated to definitively establish the protective effects of vitamin D in MS onset and clinical course and the most efficacious dose and mode of administration.

4.8 Postscript

A wealth of research has demonstrated personal UVR exposure, vitamin D intake and circulating levels of vitamin D metabolites to have significant inverse relationships with MS risk, relapse frequency and, to some extent, progression to increased disability, though further prospective studies need be done to evaluate this. These findings are in sync with the recognised immunomodulatory effects of 1,25-dihydroxyvitamin D, which in acting to shift the immune profile, both cellular and cytokine, to a less inflammatory, Th2 setting, could serve to moderate the pathways leading to neuroinflammation and clinical symptoms. The evidence from the epidemiology and molecular biology studies thus far are sufficiently supportive of a protective role for vitamin D that prospective randomised controlled trials of vitamin D as a treatment for MS clinical course should be undertaken, both independently and as adjuvant therapy with other MS therapies, and happily such studies are in progress.

In addition to its direct immunomodulatory effects in MS, vitamin D might affect the causal route by which some other pathways, including acute infection, pregnancy, stress and smoking might be mediating their effects. Additionally, other pathways which likely act independently to manifest in disease and clinical course, such as EBV and HHV-6 reactivation and childhood infections, as well as potential independent effect pathways of smoking, stress, and pregnancy, might in turn be exacerbated by low levels of vitamin D, the immunomodulatory functions of which might attenuate these other effects.

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Appendix 4A. Publication of "The varied mechanisms of vitamin D in the onset and clinical course of MS: potential roles in modulating other etiological pathways"

Simpson, Jr. SL, Greenhill K, van der Mei I, Stankovich J, Charlesworth J, Taylor B. "The varied mechanisms of vitamin D in the onset and clinical course of MS: potential roles in modulating other aetiologic pathways." *Current Medical Literature – Neurology*. 27(1) 1 – 14.

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Chapter 5. Higher 25-hydroxyvitamin D is associated with lower relapse risk in multiple sclerosis

5.1 Preface

As described in the preceding review chapter, vitamin D has a potent role to play in moderating MS clinical course. One of the major prospective cohort studies demonstrating a significant inverse relationship between circulating levels of the diagnostic metabolite of vitamin D, 25-hydroxyvitamin D and subsequent risk of relapse, is described in this chapter.

Previously, using the prospective cohort from the MS Longitudinal Study, our group demonstrated in 2008 a significant inverse relationship between biannually-measured personal UVR exposure and vitamin D levels and aggregate-level relapse rate. This chapter describes my work beyond this 2008 work, wherein I used the MS Longitudinal Study cohort to examine the relationship between serum levels of the diagnostic vitamin D metabolite 25-hydroxyvitamin D and the subsequent hazard of relapse. In addition to the as-measured biannual levels of 25-hydroxyvitamin D, I was also able to apply statistical modeling to deseasonalise 25-hydroxyvitamin D, so as to remove the seasonal variation and evaluate levels at common points in time at the season transition dates. Also, I applied similar modeling to estimate levels of 25-hydroxyvitamin D at more frequent intervals, allowing a more representative level of 25-hydroxyvitamin D around the time of relapse, and a more definitive association of vitamin D with the subsequent hazard of relapse. This chapter has been published in Annals of Neurology (ERA 2010 A*) (Appendix5A). The methods note expands on the methods of the MS Longitudinal Study, which were reported as a shorter version in the publication.

5.2 Introduction

Multiple sclerosis (MS) is a chronic central nervous system disorder characterized in the majority of cases by relapsing-remitting inflammatory demyelination on a background of slowly progressing neurodegeneration. MS has a highly variable inter- and intra-personal clinical course, both in pattern and rate of deterioration.(1) This suggests multiple contributory factors, including genetic and lifestyle determinants. However, few factors have been identified that precipitate the onset of relapses in people with MS.

One of the most striking features of MS epidemiology is that increasing latitude correlates with increasing prevalence and incidence.(2, 3) One explanation for this latitudinal gradient is the decrease in winter sunlight with increasing latitude.(4) A growing body of work(5-8) now indicates that sunlight and vitamin D may be involved in the aetiology of MS.

Vitamin D is produced in the skin by ultraviolet radiation (UVR), is found in certain foods, and may be taken as a supplement.(9) The active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)D), is critical for bone metabolism, but also has important immunomodulatory properties(10), effecting a reduction in pro-inflammatory immune pathways.(11, 12) The major circulating form, and that used to measure vitamin D, is 25-hydroxyvitamin D (25(OH)D).(13)

There is substantial epidemiological evidence, including prospective cohort studies, indicating that increased levels of sun exposure(5), larger vitamin D dietary intake(8) or higher levels of serum 25(OH)D(6) are associated with a lower risk of MS onset. However, the effect of vitamin D on clinical course is less clear. In population-level studies, correlations have been observed between the seasonal variation in levels of 25(OH)D and gadolinium-enhancing MRI lesions(14) or monthly relapse rates.(15) Three other studies(7, 16, 17) found that 25(OH)D levels were significantly lower in patients

during a relapse compared to patients with stable MS, but it is unknown whether relapses were the result or the cause of the observed low vitamin D levels. A retrospective cohort study found that higher 25(OH)D levels were associated with a lower relapse rate in the previous two years.(18)

Here we examine for the first time whether increasing levels of serum 25(OH)D are associated with a lower risk of relapse using a prospective cohort study design.

5.3 Methods

5.3.1 Study Design

The Southern Tasmanian Multiple Sclerosis Longitudinal Study was designed as a prospective longitudinal cohort study to evaluate the role of personal UVR exposure and 25(OH)D on the clinical course of MS. This study followed a cohort of 203 persons with clinically-definite MS (using 2001 McDonald criteria(19)) living in Southern Tasmania, Australia from 2002 to 2005. An estimated 78% (203/259) of eligible cases in the region were included. The study retention rate was 90% (183/203), with 4% (8/203) withdrawing early and 6% (12/203) lost because they moved interstate or died.

146 participants had a relapsing-remitting(20) (RRMS) course of MS. One person did not have any measures of 25(OH)D, leaving 145 persons included in this analysis.

Methods note 5.1 MS Longitudinal Study methods

The MS Longitudinal Study (MSL) was designed as a prospective cohort study to evaluate the environmental and genetic factors associated with MS clinical course, particularly the role of personal UVR exposure and vitamin D levels. Of the estimated 259 persons with clinically-definite MS who could participate and were living in Southern Tasmania during the period of recruitment (January 2002 – December 2004), ultimately 203 (78%) were recruited. Of these, one person did not sufficiently participate to provide useful information, and four were later re-diagnosed to a non-MS condition and removed from the study, leaving 198 participants.

The majority of participants were recruited in Winter 2002 (72.2%), with the remainder distributed as in Methods note 5.1 Table A:

Methods note 5.1 Table A. Distribution of entrance review, by sex and total.

	Males	Females	Total
Summer 2002	4 (6.6)	13 (9.5)	17 (8.6)
Winter 2002	48 (78.7)	95 (69.3)	143 (72.2)
Summer 2003	4 (6.6)	11 (8.0)	15 (7.6)
Winter 2003	2 (3.3)	7 (5.1)	9 (4.6)
Summer 2004	2 (3.3)	10 (7.3)	12 (6.1)
Winter 2004	1 (1.6)	1 (0.7)	2 (1.0)
Total	61	137	198

The majority (92.4%) of the 198 participants were followed through to the end of the study, with only 8 (4.0%) withdrawing early and 12 (6.1%) lost to follow-up when they moved overseas or died. The mean follow-up time of the cohort was 2.2 years (Median: 2.5, IQR: 2.0 – 2.6). Follow-up time did not differ by sex (p=0.80), age (p=0.14) or MS type (p=0.84).

As in Methods note 5.1 Figure A, at study entry, participants completed a questionnaire including information on their demographics and past and recent environmental exposures and behaviours (including employment, education, hobbies and activities outside, chemical exposures, physical activity, sun sensitivity and exposures/behaviour relative to the sun, allergies, medical and surgical history of themselves and their family, lifetime/childhood infections, immunisations, medications and vitamin supplements, sleep quality and quantity, pets, and alcohol, tobacco and marijuana intake) and a food frequency questionnaire. Women completed a separate questionnaire including past and recent pregnancies and post-partum behaviour, peripartum relapse occurrence, menstruation, menopause, gynaecological surgeries, hormone replacement therapy, and contraception.

Participants were assessed for a number of outcomes measures of their neurological and physiological status, including measures of disability by the Kurtzke Expanded Disability Status Scale (EDSS), the Multiple Sclerosis Severity Score (MSSS), the Scripps Neurological Rating Scale (Scripps) and the Multiple Sclerosis Functional Composite (MSFC), measures of fatigue using the Fatigue Assessment

Form (FAF), and measures of anxiety and depression using the Hospital Anxiety and Depression Scale (HADS). EDSS, MSSS, Scripps and MSFC were done at study entry and each winter follow-up review by a single study physician. FAF and HADS were done at study entry and each follow-up review.

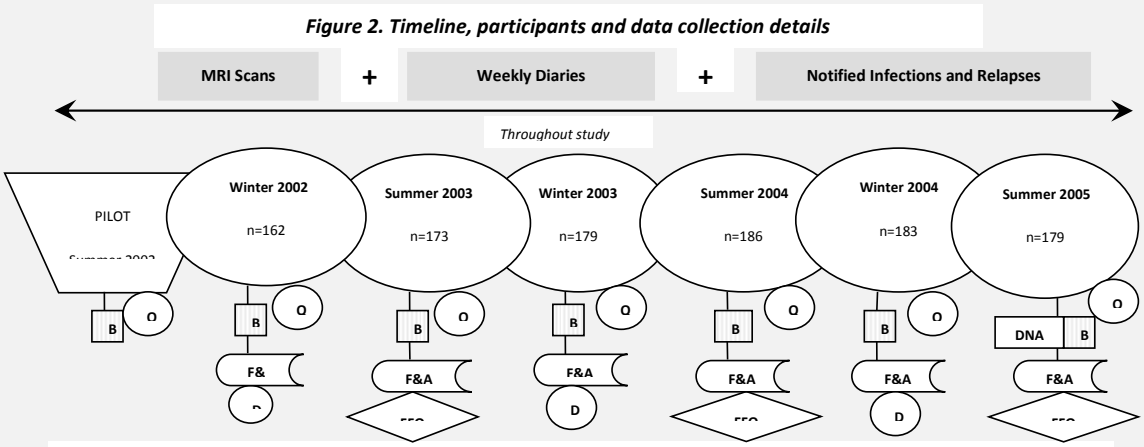
All participants were given a diary to be completed weekly with information regarding acute infections, changes in medication or vitamin supplements or immunisations, changes in MS symptoms, and for females, menstruation information.

Participants were asked to report by telephone to a study nurse when they thought they were experiencing a relapse. Additionally, at biannual review, participants were queried for the occurrence of relapse in the preceding interval since the last review. These reports were validated by the study nurse, further validated by the study physician and study neurologist.

At study entry and each biannual review, a sample of blood was obtained from participants. One aliquot was used to measure serum 25-hydroxyvitamin D – this was done at each biannual update, and at study entry for all participants except those entered in summer 2002. Serum anti-HHV-6 IgG, anti-EBV-EBNA IgG and anti-EBV-VCA IgG were measured in sample collected at study entry from all persons. Serum anti-HHV-6 IgM and serum anti-EBV-EA IgG were measured from all serum samples collected from all reviews. Serum viral load for HHV-6 and EBV were measured from all serum samples collected from all reviews. DNA from buffy coats from samples collected at study entry were used to genotype for a number of relevant genes, including HLA-DRB1, IL-10, TNF-α, and CTLA-4.

Melanin density at typically sun-unexposed sites, including the upper inner arm and the upper buttock, and at typically sun-exposed sites, including the hand and the upper shoulder, were measured using a spectrophotometer.

Methods note 5.1 Figure B. MSL study protocol structure over reviews.



Throughout study

Weekly Diaries:	Diaries included: exposure to infections, medication and supplement history, immunotherapy use, symptoms related to MS, record of menstruation.
Notified infections:	Phone questionnaire included: symptoms of infection - date first noticed, duration; nature; medical treatment; MS symptoms.

The study methodology has been previously described.(15, 21) Briefly, at each biannual review participants were asked about their lifestyle, including physical activity, whether they smoked, used immunomodulatory therapy, took vitamin D supplements, or were pregnant. At each review, a serum sample was taken for measurement of 25(OH)D levels. Clinical disability was measured every winter by a single physician, including the Expanded Disability Status Scale (EDSS). Participants completed a weekly diary, recording the occurrence of acute infection, changes in their neurological symptoms or immunomodulatory therapy, and onset of pregnancy.

Ethics approval was obtained from the Southern Tasmania Human Research Ethics Committee; all participants provided informed consent.

5.3.2 Measurement of relapses

In line with other studies, a relapse was defined as the acute or sub-acute appearance or reappearance of a neurological abnormality (lasting at least 24 hours), immediately preceded by a stable, improving or slowly progressive neurological state for 30 days, in the absence of fever, known infection, concurrent steroid withdrawal, or externally-derived increases in body temperature.(19) Using a real-time relapse notification system, participants telephoned the study centre if they thought they were experiencing a relapse – 82 relapses were reported in this fashion. Additionally, at each biannual review participants were queried about the occurrence of a relapse in the preceding six-months – 63 relapses were recorded in this manner. To ensure each relapse was a true relapse, the study nurse or physician administered a relapse questionnaire detailing relapse symptoms, medical practitioner review, treatment, and co-occurrence of infection or fever. For quality control, the study physician also performed a physical examination for those reporting a relapse by phone in year one. Very few of these (2/35 relapses) were not true relapses and these two would have been identified as such using the relapse questionnaire, validating its effectiveness. Throughout the study, each relapse was reviewed rigorously by the study physician and further by the study neurologist. After study completion, both study physician and neurologist scrutinized all relapse data, excluding events that did not fulfill the strict relapse definition. In total, five relapses were excluded in this manner; six additional relapses were excluded for being duplicates and four for occurring outside the study period. This left 122 validated relapses for use in the analysis.

5.3.3 Measurement of personal sun exposure and skin type

At each biannual review, participants estimated how much time they spent in the sun during weekends and holidays. Additionally, participants quantified to the nearest quarter-hour how much time they spent per week in the current and preceding season engaged in various activities outside. Outdoor times were summated to yield a value of the total "time outside" per week each season.

Polysulphone badges were used to measure personal UVR objectively.(22, 23) In summer and winter, participants wore one badge on Saturday and one on Sunday, placed on the outer clothing in the chest region. The badges were analyzed in the UVR laboratory at the Australian Radiation Protection and Nuclear Safety Agency.

Skin melanin density was measured on the upper inner arm using a spectrophotometer as described elsewhere.(5)

5.3.4 Measurement of 25(OH)D

Serum 25(OH)D levels were measured with a commercially available radioimmunoassay (Stillwater, Minnesota-DiaSorin Inc), which has a detection range of 12.5 to 250 nmol/L. Inter-batch reproducibility was 4.6% at 32 nmol/L and 6.4% at 125 nmol/L. All samples were stored at -80°C and shielded from light.

Samples were taken at each biannual review but all quantification of 25(OH)D levels were performed in sequential batches in order of blood draw following the conclusion of the study. Consequently neither participants nor study personnel were aware of the participants' 25(OH)D levels during the study.

5.3.5 Statistical analysis

5.3.5.1 Seasonal pattern of 25(OH)D

The seasonal pattern of 25(OH)D was modeled using methods described previously.(24) Briefly, the sinusoidal regression model was:

$$y_t = \beta_0 + \beta_1 \sin\left(\frac{2\pi t}{365}\right) + \beta_2 \cos\left(\frac{2\pi t}{365}\right)$$

where y_t denotes measured serum 25(OH)D concentration, t denotes the day of the year the sample was collected, and β_j $(j = 0,1,2)$ are estimated regression coefficients. Excluded from the sinusoidal regression model were those measurements made following a significant period of travel to a location of differing ambient UVR (n=60 measurements) and all measurements on subjects with levels of disability precluding their going outside (n=30 measurements).

Two uses were made of the fitted model. It was used to define a "summer season" of highest mean 25(OH)D levels and a "winter season" of lowest mean 25(OH)D levels. Based on the fitted model, the highest and lowest 25(OH)D mean concentrations occurred on the 19th of February and 19th of August. Therefore, summer season was defined as the period from 20 November until 19 May, and winter season as the period from 20 May until 19 November. 25(OH)D-determined season was used rather than calendar season as it was postulated to more accurately reflect the correlates of season most relevant to MS.

The model was also used to predict 25(OH)D concentrations for subjects at times of the year other than the measurement date. The predicted value at any point in time was the estimated mean 25(OH)D concentration of all subjects at that time plus the difference between the subject's last 25(OH)D measurement and the estimated mean 25(OH)D level of all subjects at the time of that measurement.

Three different models were used to account for exposure-disease temporality:

The *as-measured* model in which each subject was assigned the measured value of 25(OH)D from the day of measurement until the day of the next measurement of 25(OH)D;

The *seasonal* model in which the measured 25(OH)D concentration of each subject in summer was used to estimate that subject's 25(OH)D concentration on the preceding 20 November and

the measured 25(OH)D concentration of each subject in winter was used to estimate that subject's 25(OH)D concentration on the preceding 20 May. The 25(OH)D time-varying covariate for that subject was then assigned the summer season estimated value from 20 November until 19 May, and the winter season estimated value from 20 May until 19 November. This was done because 25(OH)D levels at Tasmania's latitude (approximately 40-44°S) show a strong seasonal variation and the fieldwork for each review was spread over three months – by correcting to a common time point, this variation was removed;

The *monthly* model in which the 25(OH)D of each subject was estimated at 30-day intervals. Given the half-life of 25(OH)D in serum has been estimated to be between 20(25) and 90(26) days, this model was designed to provide a more accurate estimate of 25(OH)D over time.

Consider an individual with a measured 25(OH)D concentration of 72 nmol/L when measured on 10 January, when the average for all subjects on 10 January was 70 nmol/L and a concentration of 50 nmol/L on 23 July, when the average was 40 nmol/L. In the as-measured analysis, this person would be assigned a 25(OH)D level of 72 nmol/L from 10 January until 23 July. Also, consider a summer season stretching from 20 November, when the cohort average value of 25(OH)D was 52 nmol/L, until 20 May of the next year. In the seasonal analysis, this person would be assigned a 25(OH)D value that was 2 units above the cohort average value on 20 November, from this date until 20 May. In the monthly analysis, the fact that this person was 2 nmol/L above the average on 10 January and 10 nmol/L above the average on 23 July was used to estimate the concentrations for each month between 10 January and 23 July.

We assessed serum 25(OH)D as a continuous term indicating linear risk. We found this was appropriate by comparing the fit of each model, with the log-likelihood ratio statistic suggesting the linear form fitted the model best.

5.3.5.2 Seasonal pattern of UVR

The same regression function described above was used to estimate personal UVR exposure at the monthly level using polysulphone badge data. Excluded from this regression model were those measurements where the participants' reported time spent outdoors was significantly different from their usual (n=312 measurements), or where participants wore the badge incorrectly (n=17 measurements).

5.3.5.3 Data analysis

The effect of 25(OH)D and other covariates, including use of immunomodulatory therapy during the study, smoking, and pregnancy, on time-to-relapse was calculated using Cox proportional hazards models for repeated events, using the time-gap model described by Prentice and colleagues(27), where multiple relapses by the same persons are treated as independent observations and the time until a prior event does not influence the composition of the risk set for a subsequent event.

All covariates satisfied the proportional hazards assumption with the exception of the binary variable for sex. For this reason, univariable results are not reported for sex and all multivariable models are stratified to allow the baseline hazards to differ by sex.

Multivariable models were adjusted for age. Survival proportions are depicted using Kaplan-Meier survival curves showing time to relapse, where multiple relapses by the same persons are treated as independent observations.

The significance of the difference in mean 25(OH)D between different subgroups was assessed using marginal models estimated by generalized estimating equations. For the analysis of the determinants of 25(OH)D, estimated monthly 25(OH)D levels were applied. The results were similar to those found when biannually-measured 25(OH)D levels were used.

All analyses were performed using STATA/SE for Windows (Version 10.1; StataCorp LP College Station, TX USA).

5.4 Results

5.4.1 Participant characteristics

The cohort of 145 participants with RRMS was followed for an average of 2.3 (SD: 0.6) years. A total of 122 confirmed relapses occurred in 70 participants (mean: 0.37 relapses per person/year). The mean 25(OH)D was 41.2 nmol/L in winter and 74.8 nmol/L in summer. During the study, 77/145 (53.1%) took a vitamin D supplement; of these only 23/77 (29.9%) took over 400 IU/day. Other features of the cohort are shown in Table 5.1.

Table 5.1. Characteristics of 145 participants with relapsing-remitting multiple sclerosis in the MS Longitudinal Study cohort.

	n/N (%)	Relapses (total number)/ person-years	Relapse rate (relapses per person-year)	Mean 25(OH)D (nmol/L)
Total	145/145	122/330.2	0.37	55.0
Sex				
Female	109/145 (75.2)	100/248.1	0.40	54.8
Male	36/145 (24.8)	22/82.1	0.27	55.6
Age at study entry				
21 – 38 years	34/145 (23.5)	29/70.9	0.41	62.5
39 – 44 years	36/145 (24.8)	32/85.6	0.37	51.7†
45 – 51 years	34/145 (23.5)	26/78.0	0.33	58.7
52 – 76 years	41/145 (28.3)	35/95.8	0.36	49.2†
Relapse during study?				
Yes	70/145 (48.3)	122/167.5	0.73	51.7
No	75/145 (51.7)	0/162.8	0.00	58.8†
Any immunomodulatory therapy during study?				
Yes	119/145 (82.1)	106/276.5	0.38	55.5
No	26/145 (17.9)	16/53.7	0.30	52.2
Body mass index[a,b]				
Normal	57/145 (39.3)	43/131.7	0.33	60.4
Overweight	57/145 (39.3)	52/124.6	0.42	54.5†
Obese	31/145 (21.4)	27/73.9	0.37	46.1†
Smoker during study?				
Yes	41/145 (28.3)	35/82.9	0.42	52.8
No	115/145 (79.3)	87/247.4	0.35	55.7
		Mean (SD; Range)		
Age at study entry, years		44.8 (10.8; 21, 76)		
MS duration from diagnosis, years		6.8 (7.2; 0, 43)		
MS duration from 1st symptoms, years		11.1 (9.1; 0, 58)		
EDSS at study entry		2.8 (1.6; 0, 8.5)		

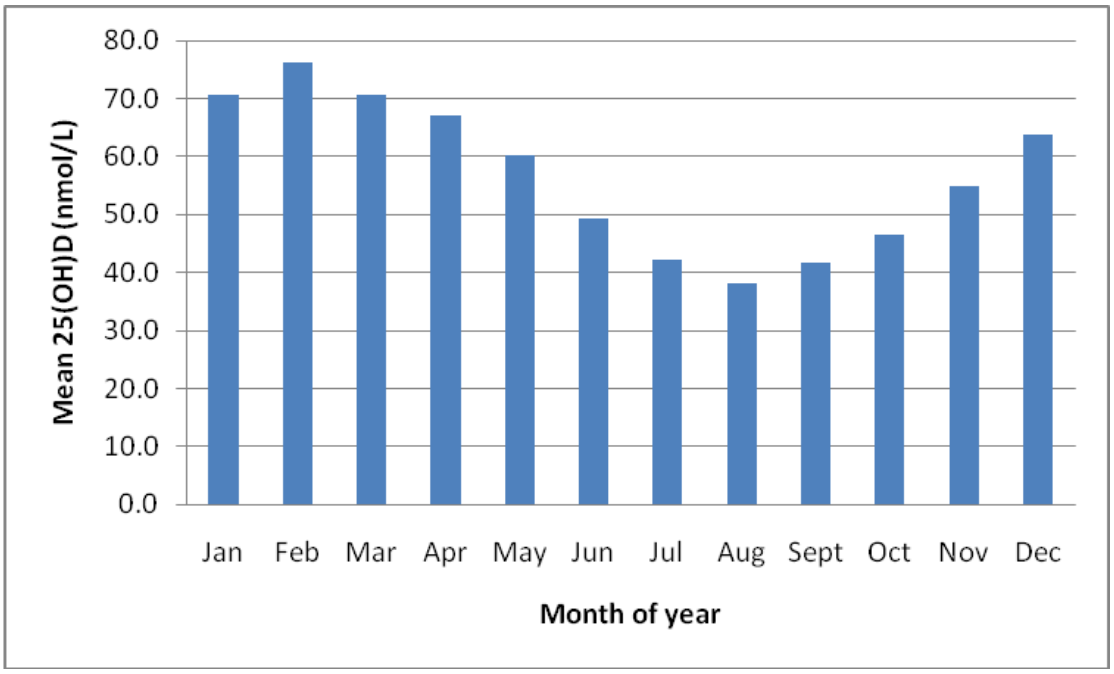
[a] Underweight=BMI<17.5, Normal=BMI 17.5-25.0, Overweight=BMI 25-29.9, Obese=BMI≥30; [b] No participants were underweight by BMI and thus this category was not included in the table †Significantly different ($p<0.05$) from initial category using random-effects generalized estimating equations.

Abbreviations: MS, multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; SD, standard deviation; EDSS, Expanded Disability Status Scale; years=years.

5.4.2 Determinants of serum 25(OH)D levels

Figure 5.1 shows the sinusoidal pattern of modelled 25(OH)D in this cohort.

Figure 5.1. Annual variation in modeled 25(OH)D by month of year, 25(OHD in nmol/L.



As expected, summer season and measures of personal sun exposure were important determinants of 25(OH)D levels. Modeling personal UVR-exposure at the monthly level increased the magnitude of the association between higher UVR exposure and subsequent 25(OH)D.

Neither female sex (Coefficient: -2.4; 95% CI: -9.5, 4.6) nor smoking status (Coefficient: 0.3; 95% CI: -2.7, 3.3) were significant determinants of 25(OH)D. Vitamin D supplement dosage was not a significant determinant of 25(OH)D (p=0.46). Physical activity was a strong predictor of 25(OH)D (Coefficient: 1.4 (95% CI: 0.8, 2.0), even after adjusting for time spent outdoors (Coefficient: 1.5 (95% CI: 0.5, 2.5). Melanin density was positively associated with 25(OH)D (Coefficient: 4.7 (95% CI: 1.7, 7.8). Age, body mass index and EDSS score were negatively associated with 25(OH)D and these effects persisted after adjustment for time spent outside (Table 5.2).

Table 5.2. Determinants of serum 25(OH)D

	Coefficient (95% CI)	p-value	Adjusted coefficient[a] (95% CI)	p-value
Season[b]				
Winter	1.0 (Reference)		1.0 (Reference)	
Summer	17.2 (16.5, 18.0)	<0.001	18.0 (16.5, 19.4)	<0.001
Time in sun (hours/day)				
<½	1.0 (Reference)			
½ - <1	1.2 (-0.7, 3.0)	0.221		
1 – <2	3.3 (1.4, 5.2)	0.001		
2 – <3	5.1 (3.0, 7.2)	<0.001		
3+	6.4 (4.1, 8.7)	<0.001		
Trend:		*<0.001*		
Time spent outside (hours/week)				
<4	1.0 (Reference)			
4 – <7	8.1 (5.4, 10.8)	<0.001		
7 – <13	11.8 (8.9, 14.7)	<0.001		
13+	18.5 (15.3, 21.6)	<0.001		
Trend:		*<0.001*		
Polysulphone-measured UVR (SEDs[c])				
<0.14	1.0 (Reference)			
0.14 – 0.39	0.6 (-1.0, 2.1)	0.463		
0.40 – 0.79	4.5 (3.0, 6.1)	<0.001		
0.80+	10.6 (9.1, 12.2)	<0.001		
Trend:		*<0.001*		
Monthly modeled UVR (SEDs)				
<0.14	1.0 (Reference)			
0.14 – 0.39	-1.6 (-3.1, -0.1)	0.040		
0.40 – 0.79	6.6 (5.1, 8.2)	<0.001		
0.80+	15.3 (13.8, 16.7)	<0.001		
Trend:		*<0.001*		
Skin melanin density (%)				
<1.00	1.0 (Reference)		1.0 (Reference)	
1.00 – 1.99	6.2 (-2.2, 14.6)	0.146	4.8 (-3.2, 12.8)	0.238
2.00 – 2.99	14.1 (6.0, 22.2)	0.001	12.4 (4.7, 20.1)	0.002
3.00+	10.7 (0.5, 20.8)	0.039	9.4 (-0.3, 19.1)	0.057
Trend:		*0.003*		*0.004*
Vitamin D supplementation in each 6-month period				
None	(Reference)		1.0 (Reference)	
<400 IU[d]/day	-0.4 (-1.9, 1.0)	0.608	0.5 (-2.1, 3.2)	0.701
400-720 IU/day	-0.9 (-3.7, 1.8)	0.500	0.3 (-4.5, 5.1)	0.914
Trend:		*0.464*		*0.777*
Physical activity (MET[e]s/day)				
0-5.9	1.0 (Reference)		1·0 (Reference)	
6.0 – 23/9	2.0 (0.5, 3.5)	0.011	2·2 (-0.5, 4.9)	0.106
24.0 – 47.9	2.8 (1.1, 4.4)	<0.001	3·7 (0.7, 7.8)	0.014
48+	4.5 (2.7, 6.3)	<0.001	4·7 (1.5, 7.8)	0.004
Trend:		*<0.001*		*0.003*

Age at study entry (years)				
0 – 39	1.0 (Reference)		1.0 (Reference)	
40 – 45	-9.9 (-17.9, -2.0)	0.014	-8.7 (-16.3, -1.1)	0.024
46 – 50	-1.9 (-10.8, 6.9)	0.667	-2.8 (-11.2, 5.6)	0.514
50+	-11.2 (-18.5, -3.8)	0.003	-11.9 (-18.9, -4.8)	0.001
Trend:		*0.020*		*0.005*
Body mass index[f,g]				
Normal	1.0 (Reference)		1.0 (Reference)	
Overweight	-6.9 (-13.4, -0.4)	0.038	-6.6 (-12.9, -0.4)	0.036
Obese	-14.5 (-22.2, -6.8)	<0.001	-13.2 (-20.6, -5.8)	<0.001
Trend:		*<0.001*		*<0.001*
EDSS[h] score at study entry				
0 – <1.0	1.0 (Reference)		1.0 (Reference)	
1.0 – <2.0	-2.6 (-13.4, 8.3)	0.645	-4.2 (-14.7, 6.2)	0.425
2.0 – <5.0	-1.5 (-12.2, 9.2)	0.787	-2.2 (-12.4, 8.1)	0.681
5.0+	-15.9 (-27.9, 3.9)	0.010	-14.2 (-25.7, 2.7)	0.016
Trend:		*0.013*		*0.038*
Pregnancy				
No	1.0 (Reference)		1.0 (Reference)	
Yes	0.3 (-3.8, 4.4)	0.896	1.3 (-5.4, 8.1)	0.697

[a] Adjusted for time spent outside per season; [b] Summer season was defined as the period from 20 November until 19 May (with 19 February as its mid-point), and the winter season was defined as the period from 20 May until 19 November (with 19 August as its mid-point); [c] SED= Standard erythemal dose; [d] IU=International unit; [e]MET=Metabolic unit; [f]Underweight=BMI<17.5, Normal=BMI 17.5-25.0, Overweight=BMI 25-29.9, Obese=BMI≥30; [g]No participants were underweight by BMI and thus this category was not included in the table; [h]EDSS=Expanded Disability Status Scale

From these results, the following findings should be interpreted as relating to predominantly UVR-derived 25(OH)D stores, in keeping with previous work.(24)

5.4.3 Univariable analysis of associations with the hazard of relapse

We examined the association between specific participant characteristics and the hazard (HR) of having a relapse in the subsequent six months (Table 5.3). There was no association between the EDSS score at study entry (HR: 1.02; 95% CI 0.89, 1.17), MS duration from first symptom (HR: 0.98; 95% CI 0.96, 1.00), or pregnancy (HR: 0.88; 95% CI 0.27, 2.87) and the hazard of relapse.

Table 5.3. Univariable associations of selected factors and relapse rate/hazard, 'as-measured' analysis.

	Number of relapses/ person-years	Relapse rate (relapses/person-years)	Hazard ratio (95% CI)
25(OH)D			
<40 nmol/L	59/124.8	0.50	1.00 (Reference)
≥40 nmol/L	62/195.8	0.30	0.60 (0.43, 0.84)
25(OH)D-season[a]			
Winter	66/172.2	0.38	1.00 (Reference)
Summer	56/158.1	0.35	0.92 (0.64, 1.32)
Vitamin D supplementation in each six-month period			
None	94/242.4	0.39	1.00 (Reference)
<400 IU/day[b]	22/63.5	0.35	0.91 (0.55, 1.49)
400+ IU/day	6/22.0	0.27	0.76 (0.33, 1.74)
			Trend: p=0.47
Number of acute infections in each six-month period			
0	41/107.6	0.38	1.00 (Reference)
1	31/100.8	0.31	0.80 (0.52, 1.25)
2+	30/83.7	0.36	0.90 (0.55, 1.46)
			Trend: p=0.63
Immunomodulatory therapy in each six-month period[†]			
No	27/74.7	0.36	1.00 (Reference)
Yes	95/255.5	0.37	1.03 (0.65, 1.63)
Sex[c]			
Male	22/82.1	0.27	
Female	100/248.1	0.40	
Age at study entry			
0 – 38 years	29/70.8	0.41	1.00 (Reference)
39 – 44 years	32/85.6	0.37	0.95 (0.52, 1.74)
45 – 51 years	26/78.0	0.33	0.84 (0.46, 1.55)
52+ years	35/95.8	0.37	0.91 (0.52, 1.57)
			Trend: p=0.67
Smoker during study			
No	87/247.4	0.35	1.00 (Reference)
Yes	35/82.9	0.42	1.21 (0.76, 1.95)

[a] Summer season was defined as the period from 20 November until 19 May (with 19 February as its mid-point), and the winter season was defined as the period from 20 May until 19 November (with 19 August as its mid-point); [b] IU=International unit; [c] Sex could not be evaluated for its hazard ratio due to its violation of the proportional hazards assumption. [†]82.6% of those on immunomodulatory therapy were using a beta-interferon product.

5.4.4 Association between serum 25(OH)D levels and hazard of relapse

We examined whether 25(OH)D levels, measured as a 10-unit continuous variable every six months, were associated with the hazard of relapses. We found that increasing levels of 25(OH)D were associated with a lower hazard of relapses

(HR: 0.91; 95% CI: 0.85-0.97, p=0.006), each 10 nmol/L increase in 25(OH)D reducing the hazard by 9% (95% CI: 3-15%). When the seasonal analysis was used to standardise all measures to the start of each season, the association persisted (HR: 0.90; 95% CI: 0.83-0.98, p=0.016), each 10 nmol/L increase in 25(OH)D reducing the hazard by 10% (95% CI: 2-17). The monthly analysis slightly enhanced the association (HR: 0.88; 95% CI: 0.82-0.95, p=0.001), each 10 nmol/L increase in 25(OH)D reducing the hazard of relapse 12% (95% CI: 5-18) (Table 5.4).

The association between 25(OH)D and hazard of relapse was linear for all three models, with no evidence of a threshold effect (Figure 5.2 for monthly model). Figure 5.3 shows the survival curve for the monthly model, demonstrating that those with higher 25(OH)D levels experienced less relapses and a longer time until relapse occurrence.

Table 5.4. Association between serum 25(OH)D levels and the hazard of a relapse, using the three different models

(Hazard Ratio expressed per 10 nmol/L increments[a]).

Analysis method	25(OH)D-10, Crude Hazard Ratio (95% CI)	25(OH)D-10, Adjusted[b] Hazard Ratio (95% CI)
As-measured	0.92 (0.86, 0.98) p=0.013	0.91 (0.85, 0.97) p=0.006
Seasonal	0.91 (0.84, 0.99) p=0.028	0.90 (0.83, 0.98) p=0.016
Monthly	0.89 (0.82, 0.96) p=0.001	0.88 (0.82, 0.95) p=0.001

[a] 10-unit increment continuous 25(OH)D hazard ratios reflect the change in the hazard of relapse for each 10-unit increase in serum 25(OH)D. [b] Analyses are adjusted for age at study entry and sex.

Figure 5.2. Hazard ratios for category of 25(OH)D in 10 nmol/L increments where level of 25(OH)D is determined using the monthly model.

Analysis is adjusted for age at study entry and stratified to allow the baseline hazards to differ by sex. Size of points is proportional to the inverse of the variance (larger bubbles represent greater precision). The plots and findings were very similar for the as-measured and seasonal models (not shown).

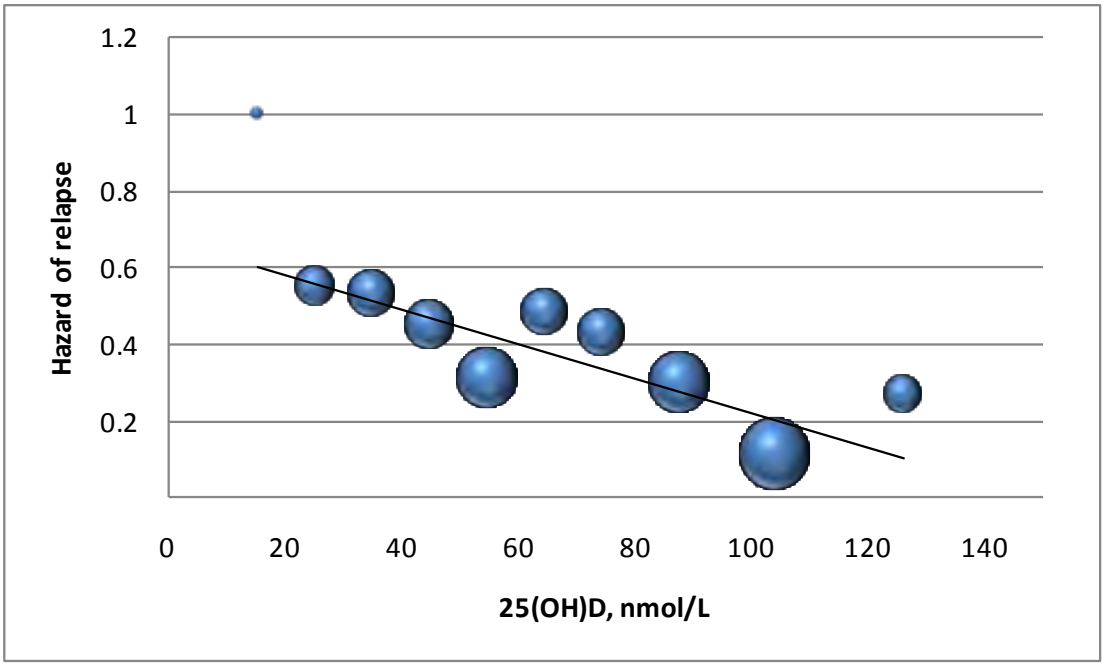
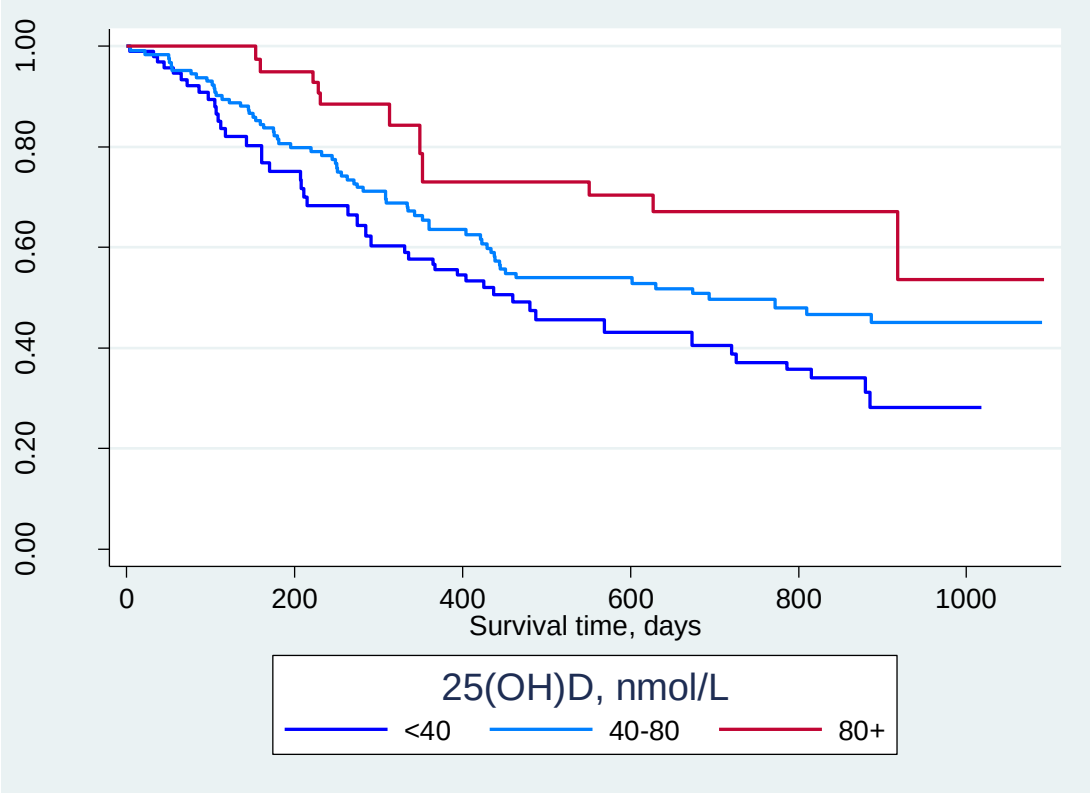


Figure 5.3. Kaplan-Meir survival plots by category of 25(OH)D where level of 25(OH)D is determined by the monthly model.

The plots show the proportion of subjects relapse-free each day since study entry. Multiple relapses by the same persons are treated as independent observations. The plots and findings were very similar for the as-measured and seasonal models (not shown).



5.4.5 Further analyses

The age and sex-adjusted association (AHR) between monthly 25(OH)D and relapse (AHR 0.88; 95% CI: 0.82-0.95) was not significantly altered by further adjustment for immunomodulatory therapy (AHR 0.88; 95% CI: 0.82-0.95), smoking (AHR 0.88; 95% CI: 0.82-0.95), higher physical activity (AHR 0.88; 95% CI: 0.82-0.95), melanin density (AHR 0.88; 95% CI: 0.82-0.95), pregnancy (AHR 0.88; 95% CI: 0.81-0.95), number of acute infections (AHR 0.90; 95% CI: 0.83-0.97), EDSS score at study entry (AHR 0.88; 95% CI: 0.82-0.95), or MS disease duration from first symptom (AHR: 0.88; 95% CI: 0.85-0.95).

The beneficial effect of higher 25(OH)D levels was evident in both winter (AHR: 0.86; 95% CI: 0.75-0.97) and summer (AHR: 0.91; 95% CI: 0.82-1.02) (p=0.45) and did not differ by the amount of personal UVR exposure, being present in both those below (AHR: 0.88; 95% CI: 0.80-0.96) and above (AHR: 0.93; 95% CI: 0.80-1.08) the mean cohort UVR (p=0.27). There was no difference between patients taking immunomodulatory therapy and those who were not (p=0.11). Importantly, even when patients with 25(OH)D insufficiency(28) (<40 nmol/L) were excluded, an inverse association remained (AHR=0.91; 95% CI: 0.81-1.01).

To examine the possibility of reverse causality, we stratified our analysis between those with an EDSS at study entry of 4.5 or less and those above 4.5. We found no significant difference (p=0.56) in the relationship between 25(OH)D and the hazard of relapse between those with lower disability (AHR: 0.89; 95% CI: 0.82, 0.96) and those with higher disability (AHR: 0.80; 95% CI: 0.63, 1.03).

5.5 Discussion

This is the first prospective study to demonstrate an association between increasing levels of 25(OH)D and a subsequent reduced hazard of relapse in people with RRMS. This relationship was dose-dependent and linear, with no evidence of a threshold effect. Importantly, this effect persisted when measurements were corrected for sampling date variations (in the seasonal analysis) and became slightly stronger when 25(OH)D estimates were used that were closer in time to a relapse (in the monthly analysis).

These associations persisted on adjustment for a diverse range of potential confounders including immunomodulatory therapy, disease course, and behavioral and environmental factors. The association was evident among those with low personal UVR exposure, suggesting the results can be generalized to locations with lower ambient summer UVR than Tasmania. Also, the association was still observed after excluding patients who were 25(OH)D insufficient, indicating that the association was not driven solely by 25(OH)D levels in the insufficiency range. There was no difference in the relationship between

25(OH)D and relapse between those with higher (EDSS>4.5) and lower (EDSS≤4.5) disability. This suggests that the association was not being driven by those with more active disease being less mobile and thus, having less UVR exposure and less 25(OH)D and argues against reverse causality as a cause of the observed relationship.

A key strength of this work is the prospective nature of our study, using repeated measures of 25(OH)D and real-time relapse notification. The size of the cohort (145 participants), the duration of follow-up (mean 2.3 years) and the comprehensive nature of the factors investigated allowed a thorough investigation of the relationship between 25(OH)D and relapse, taking into account potential confounders such as immunomodulatory therapy, smoking and seasonal covariates.

An additional strength was the use of survival analysis rather than rates – this allowed the inclusion of all information on the times of events and the change in exposure status over time, increasing temporal precision. Further, using Cox proportional hazards models for repeated events enabled inclusion of all relapses, rather than merely time to first relapse(29).

The study method was limited by the biannual collection of 25(OH)D. The ideal experiment, where the level of 25(OH)D at the time of relapse is known, is well approximated by the monthly analysis here. The modeling methods used carry assumptions about the fluctuation of 25(OH)D levels over time and assume a sinusoidal variation over time for all participants. Justification of this approach is the observation that serum 25(OH)D has been found to vary with an approximately sinusoidal pattern(30, 31) due to variation in ambient UVR and seasonal behavior. As inter-individual variation in behavior does not always allow extrapolation of this assumption to every individual, we also took into account important factors likely to affect our models, excluding those with significant disability and trips to areas of differing ambient UVR, both of which have the potential to affect the levels of 25(OH)D.

We observed up to a 12% decrease in relapse risk for each 10-nmol/L increase in serum 25(OH)D, in line with contemporaneous work by Mowry and colleagues(32) who found an inverse association between higher 25(OH)D levels and risk of relapse in pediatric-onset MS, with each 25 nmol/L increase in serum 25(OH)D reducing the subsequent relapse rate by 34%. Our data imply that increasing serum 25(OH)D levels by 50 nmol/L could more than halve the risk of relapse, a reduction at least on par with most immunomodulatory therapies.(33) Importantly, these reductions were seen in a cohort that was largely using immunomodulatory therapy (82%), suggesting that 25(OH)D has additive beneficial effects. A 50 nmol/L increase in serum 25(OH)D could be realized with supplementation of 2000 IU/day(34, 35), well below the tolerable upper limit of 10,000 IU/day.(28) Compared to immunomodulatory therapy, vitamin D-based therapies are cheaper and have much less potential for side-effects. Given that the mean 25(OH)D level in winter in this cohort was 41.2 nmol/L and that beneficial effects were observed up to 120 nmol/L, a significant reduction in relapse risk could be realized by treatment with sufficient doses of vitamin D.

There is biological plausibility for vitamin D as a protective factor against relapses. 1,25(OH)D shifts the immune response away from a pro-inflammatory profile and enhances anti-inflammatory pathways in multiple settings;(11, 12, 36) also, 25(OH)D reduces the proliferation of Th17 cells.(7) Calcitriol-fed mice have fewer clinical, histopathological and immunological signs of experimental autoimmune encephalitis, an animal model of MS(37-40).

This prospective cohort study demonstrates that for each 10 nmol/L increase in serum 25(OH)D, there was up to a 12% reduction in the hazard of relapse. These findings provide strong support for randomized-clinical trials of vitamin D-based therapies in treating relapse in RRMS.

5.6 Summary

Background: A protective association between higher vitamin D levels and the onset of multiple sclerosis (MS) has been demonstrated, however its role in modulating MS clinical course has been little studied. We investigated whether higher levels of serum 25-hydroxyvitamin D (25(OH)D) were associated with a lower risk of relapses in people with MS.

Methods: We conducted a prospective cohort study of 145 participants with relapsing-remitting MS from 2002-2005. Serum 25(OH)D levels were measured biannually and the hazard of relapse assessed using survival analysis.

Findings: There was an inverse linear relationship between 25(OH)D levels and the hazard of relapse over the subsequent six months, with HR: 0.91 (95% CI: 0.85-0.97) per 10-nmol/L increase in 25(OH)D level (p=0.006). When variation due to timing of blood collection was removed by estimating 25(OH)D at the start of each season, this association persisted, with HR: 0.90 (95% CI: 0.83-0.98) per 10-nmol/L increase (p=0.016). Taking into account the biological half-life of 25(OH)D, we estimated 25(OH)D at monthly intervals, resulting in a slightly enhanced association, with HR: 0.88 (95% CI: 0.82-0.95) per 10-nmol/L increase (p=0.001). Adjusting for potential confounders did not alter these findings.

Conclusions*:* In this prospective population-based cohort study, in a cohort largely on immunomodulatory therapy, higher 25(OH)D levels were associated with a reduced hazard of relapse. This occurred in a dose-dependent linear fashion, with each 10-nmol/L increase in 25(OH)D resulting in up to a 12% reduction in risk of relapse. Clinically, raising 25(OH)D levels by 50 nmol/L could halve the hazard of a relapse.

5.7 Postscript

This study demonstrated for the first time that there is a significant, dose-dependent relationship between serum 25(OH)D and the subsequent hazard of relapse. This association became stronger on deseasonalisation and even more so on modeling to monthly intervals, and was not abrogated by adjustment for a range of confounders, including personal UVR exposure, suggesting the effect observed is a genuine one and not a statistical artifact.

My findings here were published near contemporaneously in the same journal with a study of vitamin D as a predictor of relapse in paediatric-onset MS, which found nearly identical findings to our own (34% reduction for every 25nmol/L), despite the significantly different pathophysiology of paediatric compared to adult-onset MS, as well as the different cohort and study personnel. The robustness of the association of 25(OH)D on reducing the risk of relapse in such a range of disease types and populations is of tremendous import, since it is sufficient demonstration of this effect from associative epidemiology studies that it provides impetus for prospective, randomised controlled trials of vitamin D as a therapeutic agent in treating MS. Such trials would examine the effect of vitamin D as both an independent therapy, as well as an adjuvant to existing therapies, and the optimal dosage regimens to realise protective effects.

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Appendix 5A. Publication of "Higher 25-hydroxyvitamin D is associated with lower relapse risk in MS"

Simpson Jr. SL, Taylor B, Blizzard L, Ponsonby A-L, Pittas F, Tremlett H, Dwyer T, Gies P, van der Mei I. "Higher 25-hydroxyvitamin D is associated with lower relapse risk in MS." *Annals of Neurology*. Aug 2010; 68(2): 193-203.

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Chapter 7. Anti-HHV-6 IgG titer significantly predicts subsequent relapse risk in multiple sclerosis

7.1 Preface

In addition to its queries and measures of environmental exposures, the MS Longitudinal Study also measured personal exposures to human herpesviruses, specifically Epstein Barr Virus (EBV) and human herpesvirus 6 (HHV-6). Exposure to these viruses has been among the strongest associations with MS risk, and serological markers of exposure are found in virtually every person with MS. We sought to evaluate the relationship between measures of ever having been exposed to EBV and HHV-6 as related to MS clinical course, both the occurrence of relapse and level of disability, both at the time of measure and the change in measures of disability during the study. This chapter has been published in the journal Multiple Sclerosis (ERA 2010: A) (Appendix 7B).

7.2 Introduction

Prominent amongst the environmental factors thought to affect MS are human herpesviruses infection, particularly Epstein-Barr virus (EBV)(1, 2) and human herpesvirus 6 (HHV-6)(3), through direct(4) or indirect(5-7) mechanisms.

While there is evidence in favor of an etiological role for both herpesviruses, reported associations with disease activity are less consistent. Studies examining a relationship with clinical course have focused primarily on serological and viral load markers of viral reactivation, some demonstrating associations(8-14) but others not(9, 11, 14-16).

Given the evidence linking anti-human herpesvirus IgG levels and the risk of MS(1, 3), we hypothesised that higher levels of anti-human herpesvirus IgG may be due to a more vigorous immune response against these viral antigens, in which case there might also be an association with disease course. This has been examined to some extent previously for EBV, though results are conflicting, while for HHV-6, only two studies were found. For EBV, Farrell and colleagues(17) found a significant positive association between anti-EBV- Epstein-Barr nuclear antigen (EBNA) IgG and disease activity on MRI; additionally, persons with relapsing-remitting MS (RRMS) had significantly higher anti-EBV-EBNA IgG but significantly lower anti-EBV-viral capsid antigen (VCA) IgG levels than persons with primary-progressive MS (PPMS). Anti-EBV-EBNA IgG titers have been positively associated with CNS T2 or gadolinium-enhancing lesion number(17), and more new lesions over time(17), while in another study, serum anti-EBV-VCA IgG levels were higher in MRI-inactive than MRI-active MS(18). Anti-EBV-EBNA IgG titers have been both positively(17) and negatively(18) associated with the Expanded Disability Status Scale (EDSS), or found to have no significant correlation(19), while anti-EBV-VCA IgG titers have been found to be positively associated with EDSS(18). For HHV-6, the one study which evaluated its role in clinical course, by Villoslada and colleagues(20), found no significant difference in

the levels of anti-HHV-6 IgG between RRMS and progressive cases, in keeping with previous findings by Soldan and colleagues(21).

No studies to-date have examined the relationship between anti-HHV-6 IgG levels and the subsequent risk of relapse or change in disability. We present our findings from a longitudinal prospective cohort study in Southern Tasmania, wherein we evaluated the relationship between anti-EBV and anti-HHV-6 IgG titers and clinical outcomes in MS.

7.3 Methods

7.3.1 Study design

As described elsewhere(22), the Southern Tasmanian Multiple Sclerosis Longitudinal (MSL) Study followed over 2002-2005 a cohort of 203 persons with clinically-definite MS (2001 McDonald criteria) living in southern Tasmania, Australia. An estimated 78% (203/259) of eligible cases in the region were included, and data from 198 participants was obtained for analysis. Where participants discontinued participation or were lost to follow-up, they were censored as of the date of study exit or their last attended review.

The study methodology has been previously described(22). Briefly, at each biannual review participants were asked about their lifestyle, including physical activity, smoking, alcohol and marijuana use, sleep quality/quantity, immunisations in the preceding 6-months, vitamin D supplement use and dosage, and immunomodulatory medication use. Clinical disability was measured each winter by a single physician, including EDSS and the Multiple Sclerosis Severity Score (MSSS).

Ethics approval was obtained from the Southern Tasmania Human Research Ethics Committee; all participants provided informed consent.

7.3.2 Measurement of relapses

The measurement of relapse has been described in more detail elsewhere(23). Briefly, relapses were reported in real time by phone to the study clinician or subsequently at biannual review and all relapse reports were validated by the study physician and neurologist.

7.3.3 Biological samples

Serum samples were collected at study entry and at each biannual review and stored at -80°C until use.

Anti-HHV-6, anti-EBV-EBNA, and anti-EBV-VCA IgG titers were measured from samples collected at study entry. Serum anti-HHV-6 IgG titers were measured in parallel using an indirect immunofluorescence assay (IFA) (Panbio Inc, Columbia, MD, USA) according to the manufacturer's instructions. Dilution series were performed to obtain titers, by a single operator, at the same time in batches, and using positive and negative controls. Serum anti-EBV (VCA and EBNA) IgG titers were done using an analogous assay (Sigma-Aldrich, Castle Hill, NSW, Australia).

One subject's results for anti-EBV-EBNA IgG were indeterminate, and thus analysis of this is restricted to 197 persons.

Serum 25-hydroxyvitamin D (25(OH)D) levels were measured as previously described(23), using a commercially-available radioimmunoassay (Stillwater, Minnesota-DiaSorin Inc).

All biological measures were done after the completion of the study and thus, study investigators were blinded to these covariates when outcome data including relapse occurrence and measures of disability were recorded.

7.3.4 Statistical analysis

The distribution of anti-human herpesvirus IgG titers were left skewed. Therefore, where anti-human herpesvirus IgG titer was the dependent variable, the log base 4 transformation was used, such that each increase in titer increases the magnitude by 1.0.

The lowest stratum of each anti-human herpesvirus IgG titer had insufficient numbers to be a reference category for categorical regression analyses. Consequently, the next highest stratum was used for anti-HHV-6 IgG (40) and anti-EBV-VCA IgG (640) titers, and the next highest stratum was used for anti-EBV-EBNA IgG (40) titers.

Analysis of the factors associated with anti-HHV-6 and anti-EBV IgG titers at baseline was done using linear regression.

7.3.4.1 Survival analysis

The effect of anti-human herpesvirus IgG titers and other covariates on time-to-relapse was calculated using Cox proportional hazards models for repeated events, as described previously(23), where multiple relapses by the same persons are treated as independent observations but accounted for at the intra-individual level and the time until a prior event does not influence the composition of the risk set for a subsequent event.

All covariates satisfied the proportional hazards assumption with the exception of the binary variable for sex and the categorical variable for baseline EDSS (0-<3, 3-<5.5, 5.5-<7.5, 7.5-9). For this reason, all models are stratified to allow the baseline hazards to differ by sex and baseline EDSS category.

Survival proportions are depicted using Kaplan-Meier survival curves showing time to relapse, where multiple relapses by the same persons are treated as independent observations.

7.3.4.2 Disability and progression analyses

Factors associated with baseline-measured disability were assessed by linear regression. Predictors of mean annual change in disability were assessed by multilevel mixed-effects linear regression to account for intra-individual course over time. All analyses were adjusted for age, sex and a dichotomous term (0/1) indicating whether a person was having a relapse at the time of disability score measure; predictors of mean annual change in EDSS were also adjusted for a categorical term for baseline EDSS as

above. Transformation was applied as required to satisfy homoscedasticity; however all coefficients are reported on the scale of the original disability measure.

For survival analysis and analyses of predictors of disability and progression, subgroup analyses were done by stratification, and the significance of differences between groups, as well as the significance of multiplicative interaction between covariates, was evaluated by product terms between the corresponding variables.

For all instances where data was missing, analyses were restricted to persons with complete data.

All analyses were performed using STATA/SE for Windows (Version 10.1; StataCorp LP College Station, TX USA).

7.4 Results

7.4.1 Participant characteristics

The cohort of 198 persons was followed for an average of 2.2 (SD: 0.5) years. Of those with RRMS at study entry followed beyond one review (145), a total of 122 confirmed relapses occurred in 70 participants (mean: 0.37 relapses per person/year). Follow-up time did not differ by relevant exposure and outcome variables (data not shown). Table 7.1 shows the characteristics of the total cohort and those with RRMS at study entry.

Table 7.1. Demographic and clinical characteristics of the total cohort and those with relapsing-remitting MS at study entry

	Total cohort	RRMS sample
	n (%)	n (%)
Total	198	145
Female	137 (69.2)	109 (75.2)
Age at study entry (years)		
21 – 38	37 (18.7)	34 (23.5)
39 – 44	40 (20.2)	36 (24.8)
45 – 51	45 (22.7)	34 (23.5)
52 – 77	76 (38.3)	41 (28.3)
MS course at study entry		
RRMS	149 (75.3)	145 (100)[a]
SPMS	40 (20.2)	0
PPMS	9 (4.6)	0
Progression to SPMS during study	17 (11.4)	17 (11.7)
Relapse during study	70 (35.4)	70 (48.3)
Any immunomodulatory therapy during study?	130 (65.7)	119 (82.1)
Smoker during study?	49 (24.8)	39 (26.9)
anti-HHV-6 IgG titer		
10	11 (5.6)	9 (6.2)
40	58 (29.3)	39 (26.9)
160	90 (45.5)	69 (47.6)
640	39 (19.7)	28 (19.3)
anti-EBV-EBNA IgG titer[b]		
<10	5 (2.5)	4 (2.8)
10	10 (5.1)	8 (5.5)
40	32 (16.2)	23 (15.9)
160	115 (58.4)	82 (56.6)
640	35 (17.8)	28 (19.3)
anti-EBV-VCA IgG titer		
160	20 (10.1)	13 (9.0)
640	119 (60.0)	90 (62.1)
2560	59 (29.8)	42 (28.9)
	Mean (SD; Range)	
Age at study entry, years	48.2 (11.4; 21 – 77)	45.5 (10.4; 21 – 76)
MS duration from diagnosis, years	9.2 (8.8; 0 – 43)	6.9 (7.3; 0 – 43)
MS duration from 1st symptoms, years	14.0 (10.3; 0 – 58)	11.3 (9.2; 0 – 58)
EDSS at study entry	3.7 (2.3; 0 – 9)	2.8 (1.6; 0 – 8.5)
EDSS at study exit	4.3 (2.2; 0 – 9.5)	3.5 (1.7; 0 – 8.5)
MSSS at study entry	4.5 (2.7; 0.1 – 9.9)	3.8 (2.4; 0.1 – 9.9)
MSSS at study exit	5.0 (2.6; 0.1 – 9.9)	4.3 (2.4; 0.1 – 9.9)

[a] Only 145 of the 149 persons with RRMS at study entry are used in the relapse analysis since the other four persons only completed one review, precluding survival analysis of the occurrence of relapse. [b] One person did not have a value for anti-EBV-EBNA IgG.

Abbreviations: MS, multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; SD, standard deviation; EDSS, Expanded Disability Status Scale; MSSS, Multiple Sclerosis Severity Score; Scripps, Scripps Neurological Rating Scale; MSFC, Multiple Sclerosis Functional Composite; HHV-6=human herpesvirus 6; EBV=Epstein-Barr virus; EBNA=Epstein-Barr nuclear antigen; VCA=viral capsid antigen; IgG=immunoglobulin class G; EDSS=Expanded Disability Severity Scale; MSSS=Multiple Sclerosis Severity Score.

7.4.2 Determinants of anti-HHV-6 and anti-EBV IgG titres

Anti-HHV-6 IgG showed no correlation with either anti-EBV-EBNA IgG (r=0.09, p=0.23) or anti-EBV-VCA IgG (r=0.05, p=0.53); similarly on restriction to persons of RRMS course followed beyond baseline there were no correlations (HHV6-EBNA: r=0.08, p=0.34; HHV6-VCA: r=0.04, p=0.68).

Anti-HHV-6 IgG titers were 1.5-times higher among females compared to males (p=0.020). On restriction to persons of RRMS course at study entry, this effect was not evident (p=0.51). Neither anti-EBV-VCA nor anti-EBV-EBNA IgG titers differed by sex. The reduction in magnitude of the effect of sex on anti-HHV-6 IgG titer on restriction to RRMS occurred because females with secondary progressive MS (SPMS) and PPMS had significantly higher titers than the males (2.8-times higher, p=0.001), this persisting on adjustment (p=0.005). Among females, anti-HHV-6 IgG titers were 2.0-times higher among progressive course relative to RRMS, though this did not reach statistical significance (p=0.14). Among females who were RRMS at baseline, there was no significant difference in anti-HHV-6 IgG titer among females who progressed to SPMS during the study compared to those who did not (p=0.84). Interestingly, males trended in the opposite direction from females, with 1.6-times higher anti-HHV-6 IgG titers in RRMS than progressive course cases (p=0.12), though this was attenuated on adjustment (p=0.20); like females, males showed no association with progression to SPMS after adjustment (p=0.44).

Among persons able to have relapses, i.e. persons of RRMS course at study entry, those reporting a relapse during the study have 1.7-times higher titers of anti-HHV-6 IgG than those who did not have relapses (p=0.004). This association remained after adjustment (adjusting for age, sex, MS course at study exit, serum 25(OH)D and season at time of anti-human herpesvirus IgG measure, baseline

disability, MS duration from diagnosis, and use of immunomodulatory medication during the study) (p=0.015).

Other covariates examined in Appendix 7A Tables 1 and 2, including age, BMI, duration of disease, 25(OH)D at time of anti-human herpesvirus IgG measure, smoking behaviour and use of immunomodulatory therapy during the review showed no association with any of the anti-EBV IgG titers, either overall or on restriction to RRMS participants.

7.4.3 Anti-HHV-6 and anti-EBV IgG titres and relapse

We found a strong, dose-dependent association between baseline-measured anti-HHV-6 IgG and the subsequent hazard of relapse. This association was not affected by adjusting for anti-EBV-EBNA and anti-EBV-VCA IgG (Table 7.2, panel A), nor by adjusting for potential confounders, including exit course, duration of disease from diagnosis, season at time of anti-human herpesvirus measure, 25(OH)D at time of anti-human herpesvirus measure, body mass index, and use of immunomodulatory therapy during the study. The association did not differ by sex (p=0.92).

Table 7.2. Association between baseline anti-HHV-6 IgG titers and the hazard of relapse for the full duration (Panel A), and restricted to first year and first six-months after measure of anti-HHV-6 IgG titers (Panel B). All models adjusted for age and stratified by sex and baseline EDSS category.

A	Full study interval n=122		
	Crude HR (95% CI)	Adjusted[a] HR (95% CI)	Adjusted for confounders[b] HR (95% CI)
HHV-6 IgG titer			
10	1.00 [Reference]	1.00 [Reference]	1.00 [Reference]
40	1.17 (0.29, 4.80)	1.18 (0.29, 4.75)	1.09 (0.27, 4.45)
160	1.82 (0.48, 6.94)	1.88 (0.50, 7.04)	1.49 (0.40, 5.59)
640	2.99 (0.77, 11.66)	3.10 (0.81, 11.91)	2.63 (0.66, 10.55)
Trend	***p=0.001***	***p=0.001***	***p=0.003***

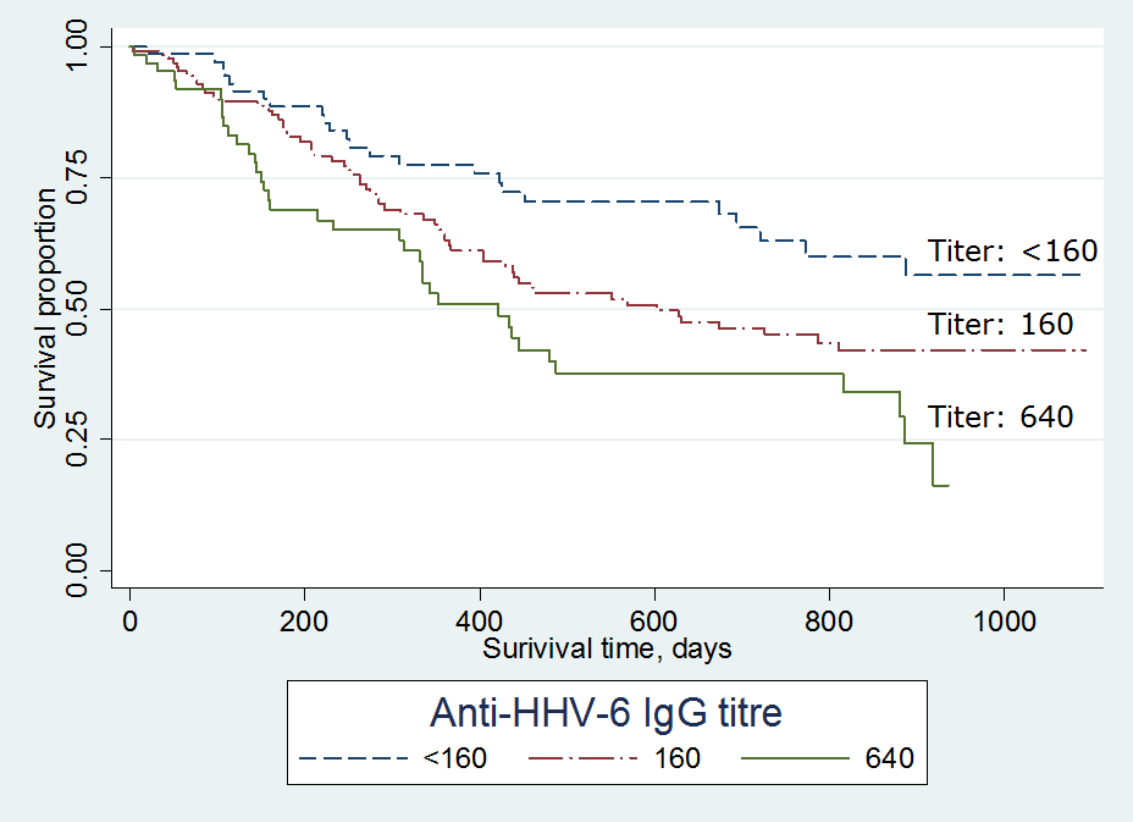
B	First year restricted (n=52)		First 6-months restricted (n=25)	
	Crude HR (95% CI)	Adjusted[a] HR (95% CI)	Crude HR (95% CI)	Adjusted[a] HR (95% CI)
HHV-6 IgG titer				
10	1.00 [Reference]	1.00 [Reference]	1.00 [Reference]	1.00 [Reference]
40	0.61 (0.17, 2.17)	0.62 (0.18, 2.17)	0.13 (0.02, 1.10)	0.13 (0.02, 1.07)
160	1.35 (0.45, 4.07)	1.43 (0.48, 4.21)	0.61 (0.22, 1.65)	0.65 (0.23, 1.81)
640	2.25 (0.69, 7.29)	2.40 (0.77, 7.52)	1.56 (0.57, 4.25)	1.66 (0.63, 4.36)
Trend	***p=0.010***	***p=0.008***	***p=0.002***	***p=0.002***

Note: Reference category for anti-HHV-6 IgG titer set at 160 titer due to smaller cell numbers in 10 and 40 categories.
[a] Further adjusted for anti-EBV-EBNA and anti-EBV-VCA IgG. [b] Further adjusted for exit course, duration of disease from diagnosis, season at time of anti-human herpesvirus measure, 25(OH)D at time of anti-human herpesvirus measure, body mass index, and use of immunomodulatory therapy during the study.

Figure 7.1 shows that the higher relapse rate among those with higher baseline anti-HHV-6 IgG remains over the full duration of the study. However, as the baseline-measured IgG may relate more to earlier than later occurring relapses, we evaluated the association restricted to the first year and six-months after measure. As in Table 7.2 (panel B), the association persists in magnitude and significance, despite the reduced numbers of relapses included (122 overall, 52 in first year, 25 in first six-months). No association was found between either of the anti-EBV IgGs in either the 1-year or 6-month restricted analyses (data not shown).

Figure 7.1. Kaplan-Meir survival plots by category of anti-HHV-6 IgG titre (labelled at end of each line).

The plots show the proportion of subjects relapse-free each day since study entry. Multiple relapses by the same persons are treated as independent observations



7.4.4 The prospective association between anti-HHV-6 and anti-EBV IgG and clinical disability progression

We estimated the association between anti-human herpesvirus IgG and baseline-measured disability, with a positive change indicating a higher level of disability. There was no association between anti-HHV-6 IgG, anti-EBV-EBNA IgG or anti-EBV-VCA and either baseline-measured EDSS or MSSS. We next estimated the annual change in disability at each antibody level, with a positive change indicating a worsening in disability. There was no evidence that higher anti-human herpesvirus IgG level was associated with higher or lower change in EDSS or MSSS (Appendix 7A Table 3). No significant differences were found by sex or by MS type (data not shown).

7.5 Discussion

Using one of the largest and longest-follow-up prospective cohorts evaluating the role of anti-HHV-6 and anti-EBV IgG in the clinical course of MS, we have demonstrated a dose-dependent positive association between baseline-measured serum anti-HHV-6 IgG titers and the subsequent hazard of relapse, persisting on adjustment for anti-EBV IgG. While no anti-HHV-6 or EBV IgG titers were significantly associated with disability, anti-HHV-6 IgG titer was differentially associated with MS course by sex.

7.5.1 Anti-HHV-6 and anti-EBV IgG and relapse

The finding of a strong, dose-dependent relationship between baseline anti-HHV-6 IgG and the subsequent hazard of relapse is an interesting and novel finding. While a possible initial interpretation is that higher titers reflect nonspecific immune response against herpesvirus, the absence of similar trends for EBV argues against this, suggesting a specific anti-HHV-6 response. It seems that the immune response is specific solely to HHV-6 antigens, or host antigens resembling HHV-6 and may connote increased risk via molecular mimicry and similarity of HHV-6-specific antigens with host antigens in the CNS(7). A further possibility is that the immune response against HHV-6 is proportionate to the level, either current or recent, of HHV-6 and that the virus is manifesting in greater disease activity through overt cytolytic replication and resultant inflammation. HHV-6 has been found in the CNS of patients with MS(25) and MS-associated lesions(26) so certainly this is possible. HHV-6 has a number of potentially etiologic effects, including latency in(27, 28) and induction of neurotoxic behaviour in some glial cells(29). HHV-6 uses the surface protein CD46 to enter cells and it has been demonstrated that this induces production of interleukin-1β and interleukin-17(30), and HHV-6 encodes a viral version of the CCR2 ligand, which acts as a chemo-attractant for monocytes and macrophages(31). Also, HHV-6 has the capacity to transactivate other latent herpesviruses and human endogenous retroviruses (HERV)(32, 33), translated components of which have been found to be pro-inflammatory(34) and neuro and gliotoxic(35).

7.5.2 Anti-HHV-6 and anti-EBV IgGs, sex and MS course

Anti-HHV-6 IgG titres in females were similar to males within the RRMS group, but among those with progressive courses of MS, females had titres that were 2.8 times higher than males. Viewed differently, progressive females had 2.0 times higher titres than RRMS females, while progressive males had 1.6 times lower titres than RRMS males, although this was not statistically significant. Anti-HHV-6 IgG titer did not differ between those that progressed to SPMS during the study compared to those who did not, overall or by sex.

We found no difference in titers of anti-EBV-EBNA or anti-EBV-VCA IgG by sex or by MS course. These findings are at odds with the results of other studies using similar methodologies(17, 20, 21, 37). Villoslada and colleagues(20) did find significantly lower anti-HHV-6 IgM detection frequencies in SPMS than MSMS, as well as lower anti-HHV-6 IgM titers in SPMS than RRMS. Despite the majority female cohort, these trends are analogous to our findings among males, and may reflect population-specific effects.

7.5.3 Anti-HHV-6 and anti-EBV IgG and disability

We found no significant association between anti-human herpesvirus IgGs and baseline-measured EDSS or MSSS, nor with mean annual change over time. Castallezzi and colleagues recently reported a positive association between anti-EBV VCA IgG and EDSS and a negative association between anti-EBV EBNA IgG, but found little evidence of anti-EBV intrathecal immune activity and concluded that immune response to EBV is unlikely to be the mediator of MS immunopathology(18). The observed positive association with VCA-specific IgG and increased disability but not with EBNA-specific IgG could reflect activity of EBV replicative proteins but not viral assembly or might reflect that EBV is not the direct causative agent, but rather is transactivating something else, possibly HHV-6 and/or HERV.

7.5.4 Strengths and weaknesses

This study is stronger relative to previous studies by virtue of its prospective design, longer follow-up time and larger cohort size. Also, studies heretofore have evaluated the role of HHVs in MS clinical

course either with a cross-sectional study design or with a longitudinal design using serial-measures within the same person(8, 10, 12-17), comparing anti-human herpesvirus measures during exacerbation and remission. Here we prospectively evaluated the role of HHVs on relapses and progression in disability.

Our cohort was similar in terms of relapse occurrence(10, 12, 14) and level of disability(12, 14, 17, 18, 20) to others studies on this topic. Thus our results are likely generalizable to other European-descent populations at a minimum.

A limitation was that anti-HHV6 IgG and anti-EBV EBNA and VCA IgG was only measured at baseline, but these antibodies are relatively stable over time(38, 39). Our analysis found the association persisted when we restricted the analysis period to 1-year and 6-months post-anti-human herpesvirus IgG measure, providing support that the observed finding reflects a true effect, rather than statistical artifact.

7.5.5 Conclusion

To conclude, using one of the largest prospective cohorts and longest follow-up durations evaluating the role of anti-HHV-6 and anti-EBV IgG in MS clinical course, we found a significant, dose-dependent relationship between baseline-measured serum anti-HHV-6 IgG levels and the subsequent hazard of relapse. At the same time, while no association was found with disability or change in disability, the higher anti-HHV-6 IgG titers among progressive-course females relative to males indicates some interaction between anti-HHV-6 IgG and sex in progressive MS course. Overall, these findings suggest that measuring anti-HHV-6 IgG levels in newly diagnosed RRMS cases may be an indicator of future disease activity and may allow stratification of high and low risk individuals after a first demyelinating event.

7.6 Summary

Background: Some of the strongest associations with MS onset are for human herpesviruses, particularly Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV-6). Their role in MS clinical course is less clear, however.

Methods: Prospective cohort of 198 persons with clinically-definite MS, followed 2002-5 and serum samples obtained from all subjects at study entry to measure anti-HHV-6 and anti-EBV (EBNA and VCA) IgG titers. Association with relapse evaluated using survival analysis; association with disability/progression using linear regression or multilevel mixed-effects linear regression.

Results: For the 145 persons with relapsing-remitting MS followed beyond one review, anti-HHV-6 IgG titer was positively associated with the hazard of relapse with a dose-dependent trend (p=0.003), not affected by adjustment for anti-EBV IgG titers, neither of which were independently associated with relapse. There was no significant association between anti-human herpesvirus IgG titers and baseline-measured disability scores, or change in disability scores; however anti-HHV-6 IgG titers were 2.8-times higher among progressive-course females relative to progressive-course males.

Discussion: These findings suggest that in addition to a potential etiological role in MS, HHV-6 infection or the immune response to HHV-6 antigens may have an effect on the risk of MS relapses and possibly on progressive courses of MS. The observed effect was directly related to anti-HHV-6 IgG titers and may indicate that either HHV-6 infection or factors associated with an altered humoral immune response to HHV-6 may have an effect on MS clinical course. Anti-HHV-6 IgG titer may be a useful prognostic factor in relapsing-remitting MS clinical course.

7.7 Postscript

This study demonstrates that, in addition to its significant association with MS risk, levels of serum anti-HHV-6 IgG strongly predicts the subsequent hazard of relapse. Additionally, there seems to be a significant interaction between serological markers of HHV-6 exposure and sex and MS course, with females of progressive MS type (SPMS, PPMS) having significantly higher anti-HHV-6 IgG than progressive course males. The mode by which this association is manifest may include a stronger immune response against HHV-6 antigens and potentially cross-reactivity with similar host antigens, or it may reflect more frequent reactivation of HHV-6 in persons with more active disease.

7.8 References

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Appendix 7A. Supplemental tables: predictors of anti-HHV IgG titres & anti-HHV IgG titres & measures of disability

Appendix 7A Table 3. Determinants of log4-transformed anti-HHV titres, all persons*. Analyses done using linear regression.

	HHV-6 IgG β (95% CI)	EBV-EBNA IgG‡ β (95% CI)	EBV-VCA IgG β (95% CI)
Sex			
Male	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Female	**0.29 (0.05, 0.54)**	0.01 (-0.27, 0.29)	0.14 (-0.04, 0.33)
	p=0.020	*p=0.95*	*p=0.12*
Age (years)			
21 – 38	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
39 – 44	-0.05 (-0.41, 0.32)	-0.22 (-0.64, 0.20)	-0.07 (-0.34, 0.20)
45 – 51	-0.32 (-0.68, 0.03)	-0.21 (-0.61, 0.20)	0.00 (-0.26, 0.27)
52 – 76	-0.18 (-0.51, 0.14)	-0.11 (-0.48, 0.26)	-0.09 (-0.32, 0.15)
Trend:	*p=0.18*	*p=0.75*	*p=0.57*
BMI			
Normal	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Overweight	0.04 (-0.24, 0.31)	0.01 (-0.30, 0.32)	**0.22 (0.02, 0.42)**
Obese	-0.19 (-0.53, 0.14)	-0.12 (-0.50, 0.26)	**0.26 (0.02, 0.50)**
Trend:	*p=0.35*	*p=0.59*	***p=0.021***
Entry course			
RRMS	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
SPMS	-0.13 (-0.42, 0.16)	-0.08 (-0.41, 0.25)	-0.08 (-0.29, 0.14)
PPMS	0.31 (-0.25, 0.86)	0.17 (-0.46, 0.80)	0.24 (-0.16, 0.65)
Trend:	*p=0.87*	*p=0.94*	*p=0.73*
Any relapse during study?			
No	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Yes	**0.39 (0.12, 0.65)**	0.10 (-0.21, 0.42)	0.08 (-0.11, 0.28)
	p=0.004	*p=0.52*	*p=0.40*
MS duration from diagnosis (years)			
<3	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
3 – <6	**0.42 (0.08, 0.76)**	0.27 (-0.12, 0.65)	0.10 (-0.15, 0.35)
6 – <14	0.19 (-0.11, 0.49)	0.04 (-0.30, 0.39)	0.21 (-0.01, 0.44)
14+	0.02 (-0.28, 0.32)	0.26 (-0.09, 0.60)	0.08 (-0.14, 0.31)
Trend:	*p=0.91*	*p=0.27*	*p=0.26*
MS duration from symptom onset (years)			
<3	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
3 – <6	0.15 (-0.18, 0.48)	0.10 (-0.28, 0.47)	-0.02 (-0.26, 0.23)
6 – 14	0.23 (-0.07, 0.54)	-0.03 (-0.38, 0.32)	0.06 (-0.17, 0.29)
14+	-0.13 (-0.45, 0.19)	0.13 (-0.24, 0.49)	-0.01 (-0.25, 0.23)
Trend:	*p=0.73*	*p=0.67*	*p=0.88*

Appendix 7A. Supplemental tables: predictors of anti-HHV IgG titres & anti-HHV IgG titres & measures of disability

25(OH)D at contemporaneous review (nmol/L)			
<27.00	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
27.00 –35.00	0.09 (-0.24, 0.42)	0.04 (-0.34, 0.41)	-0.05 (-0.29, 0.20)
35.01 – 52.00	-0.07 (-0.38, 0.25)	-0.08 (-0.43, 0.28)	-0.03 (-0.26, 0.20)
52.01+	-0.08 (-0.40, 0.24)	0.04 (-0.32, 0.40)	-0.12 (-0.35, 0.12)
Trend:	*p=0.47*	*p=0.99*	*p=0.38*
Ever smoked?			
No	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Yes	0.18 9-0.06, 0.42)	0.09 (-0.18, 0.36)	0.02 (-0.16, 0.20)
	p=0.13	*p=0.53*	*p=0.81*
Immunomodulatory therapy at review			
No	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Yes	0.11 (-0.12, 0.35)	-0.05 (-0.31, 0.22)	0.13 (-0.05, 0.30)
	p=0.35	*p=0.74*	*p=0.15*

‡Note: anti-EBV EBNA data is missing for one person for whom the assay results were indeterminate.
Abbreviations: MS=multiple sclerosis; RRMS=relapsing-remitting MS; SPMS=second-progressive MS; PPMS=primary progressive MS; HHV-6=human herpesvirus 6; EBV=Epstein-Barr virus; EBNA=Epstein-Barr nuclear antigen; VCA=viral capsid antigen; IgG=immunoglobulin class G.

Appendix 7A Table 4. Determinants of log4-transformed anti-HHV titres for RRMS sample*. Anti-HHV-6, EBV-EBNA and EBV-VCA assessed by linear regression; anti-EBV-EA assessed by generalised linear estimator models.

	HHV-6 IgG β (95% CI)	EBV-EBNA IgG‡ β (95% CI)	EBV-VCA IgG β (95% CI)
Sex			
Male	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Female	0.10 (-0.21, 0.42)	0.18 (-0.18, 0.55)	0.12 (-0.10, 0.34)
	p=0.51	*p=0.32*	*p=0.29*
Age (years)			
21 – 38	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
39 – 44	-0.11 (-0.50, 0.28)	-0.22 (-0.68, 0.24)	-0.04 (-0.32, 0.24)
45 – 51	-0.27 (-0.66, 0.13)	-0.23 (-0.69, 0.24)	-
52 – 76	-0.19 (-0.56, 0.19)	-0.07 (-0.52, 0.37)	-0.09 (-0.36, 0.18)
Trend:	*p=0.26*	*p=0.81*	*p=0.58*
BMI			
Normal	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Overweight	0.06 (-0.25, 0.36)	0.07 (-0.29, 0.43)	0.21 (-0.01, 0.43)
Obese	-0.22 (-0.59, 0.15)	-0.08 (-0.52, 0.35)	**0.31 (0.05, 0.57)**
Trend:	*p=0.33*	*p=0.80*	***p=0.012***
Progression from RRMS to SPMS during study?			
No	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Yes	0.16 (-0.26, 0.58)	-0.13 (-0.62, 0.36)	0.17 (-0.13, 0.47)
	p=0.45	*p=0.59*	*p=0.25*
Any relapse during study?			
No	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Yes	**0.39 (0.12, 0.65)**	0.10 (-0.21, 0.42)	0.08 (-0.11, 0.28)
	p=0.004	*p=0.52*	*p=0.40*
MS duration from diagnosis (years)			
<3	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
3 – <6	**0.45 (0.10, 0.81)**	0.28 (-0.14, 0.71)	0.05 (-0.20, 0.31)
6 – <14	**0.39 (0.04, 0.73)**	0.22 (-0.19, 0.64)	0.20 (-0.05, 0.45)
14+	0.05 (-0.35, 0.46)	0.22 (-0.26, 0.70)	0.22 (-0.07, 0.51)
Trend:	*p=0.26*	*p=0.25*	*p=0.06*
MS duration from symptom onset (years)			
<6	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
6 – <12	0.15 (-0.20, 0.50)	0.15 (-0.26, 0.56)	-0.05 (-0.31, 0.20)
12 - <20	0.35 (-0.00, 0.70)	-0.08 (-0.49, 0.33)	0.07 (-0.19, 0.32)
20+	0.02 (-0.40, 0.44)	0.28 (-.21, 0.78)	0.00 (-0.30, 0.31)
Trend:	*p=0.32*	*p=0.55*	*p=0.75*
25(OH)D at current review (nmol/L)			
<27.00	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
27.00 –35.00	-0.17 (-0.59, 0.25)	-0.09 (-0.58, 0.40)	-0.16 (-0.46, 0.14)
35.01 – 52.00	-0.20 (-0.58, 0.17)	0.11 (-0.33, 0.54)	-0.12 (-0.39, 0.15)
52.01+	-0.29 (-0.65, 0.07)	0.09 (-0.34, 0.51)	-0.21 (-0.47, 0.05)
Trend:	*p=0.12*	*p=0.54*	*p=0.14*

Appendix 7A. Supplemental tables: predictors of anti-HHV IgG titres & anti-HHV IgG titres & measures of disability

Ever smoked?			
No	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Yes	0.17 (-0.10, 0.44)	0.12 (-0.21, 0.44)	0.00 (-0.20, 0.20)
	p=0.22	*p=0.48*	*p=1.00*
Immunomodulatory therapy at review			
No	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Yes	0.14 (-0.18, 0.47)	-0.04 (-0.42, 0.34)	0.14 (-0.10, 0.37)
	p=0.38	*p=0.84*	*p=0.25*

[*] RRMS sample is 145 persons who entered study with a relapsing-remitting MS course who were followed beyond the initial review, allowing prospective evaluation of clinical course. [‡]Note: anti-EBV EBNA data is missing for one person for whom the assay results were indeterminate.

Abbreviations: MS=multiple sclerosis; RRMS=relapsing-remitting MS; SPMS=second-progressive MS; HHV-6=human herpesvirus 6; EBV=Epstein-Barr virus; EBNA=Epstein-Barr nuclear antigen; VCA=viral capsid antigen; IgG=immunoglobulin class G.

Appendix 7B. Publication of "Anti-HHV-6 IgG titre significantly predicts subsequent relapse risk in multiple sclerosis"

Simpson, Jr. SL, Taylor B, Dwyer D, Taylor J, Blizzard L, Ponsonby A-L, Pittas F, Dwyer T, van der Mei, I. "Anti-HHV-6 IgG titers significantly predict subsequent relapse risk in multiple sclerosis." *Multiple Sclerosis*. In-press.

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Chapter 8. Serological HHV-6 reactivation is not associated with multiple sclerosis relapse

8.1 Preface

As noted in the preceding chapter, serological evidence of HHV-6 exposure is strongly predictive of subsequent hazard of relapse in a dose-dependent fashion. This might come about by more frequent reactivation of HHV-6 in persons with more active disease, with higher anti-HHV-6 IgG merely reflecting more recent reactivation. Using serially-measured serum samples collected at biannual review during the MS Longitudinal Study, we measured anti-HHV-6 IgM, the serological marker of HHV-6 reactivation, to assess whether reactivation of HHV-6 correlated with MS clinical activity, namely relapse and greater level of disability.

8.2 Introduction

Multiple sclerosis (MS) is a progressive demyelinating condition of the central nervous system. MS is a complex disease with a number of genetic and environmental factors believed to affect its development and clinical course(1, 2). A role has been suggested for infection and/or reactivation of herpesviruses, particularly Epstein-Barr virus and Human Herpesvirus 6 (HHV-6). While some role for HHV-6 in risk of MS has been found(3, 4), its role in MS clinical course (e.g. relapse rate and disease progression to disability) is less clear.

The evidence on reactivation and MS course is mostly restricted to associations with relapses, by comparing HHV-6 activation among those with a relapse compared to those in remission. Some studies are longitudinal with serial measures(5, 6), but the sample sizes are generally small and the follow-up time short. None have measured HHV-6-specific IgM as a marker of reactivation. The results of these studies have been mixed, with some finding a positive association with HHV-6 reactivation and clinical activity(5-10) and others not(11-13).

We have evaluated HHV-6 reactivation by measuring HHV-6-specific IgM in 1050 samples in a prospective cohort of 198 people with clinically-definite MS living in Southern Tasmania, Australia (2002-2005). We examined the association between HHV-6 IgM and MS relapses and clinical progression.

8.3 Methods

As described in more detail elsewhere(14-16), we prospectively followed a cohort of 199 persons with clinically-definite MS living in Southern Tasmania from 2002-2005. Briefly, participants were evaluated biannually for a number of factors, including sun exposure, history of acute infections, smoking and fatigue. Clinical disability was measured annually (EDSS, MSSS, Scripps, MSFC).

In line with other studies, a relapse was defined as the acute or sub-acute appearance or reappearance of a neurological abnormality (lasting at least 24 hours), immediately preceded by a stable, improving or slowly progressive neurological state for 30 days, in the absence of fever, known infection, concurrent steroid withdrawal, or externally-derived increases in body temperature.(17) Using a real-time relapse notification system, participants telephoned the study centre if they thought they were experiencing a relapse – 82 relapses were reported in this fashion. Additionally, at each biannual review participants were queried about the occurrence of a relapse in the preceding six-months – 63 relapses were recorded in this manner. To ensure each relapse was a true relapse, the study nurse or physician administered a relapse questionnaire detailing relapse symptoms, medical practitioner review, treatment, and co-occurrence of infection or fever. Throughout the study, each relapse was reviewed rigorously by the study physician and further by the study neurologist, with 122 validated relapses left for analysis.

Serum was taken and stored at -80°C at each review. Anti-HHV-6 IgG titres were measured at baseline; serum anti-HHV-6 IgM titres were measured at each review and when a relapse was reported. Serum anti-HHV-6 IgG and IgM were measured in parallel using an indirect immunofluorescence assay (IFA, Panbio Inc., Columbia, MD, USA) according to the manufacturer's instructions, and dilution series performed to obtain titres, at a single sample, by a single operator, at the same time in batches, and using positive and negative controls.

A total of 1050 samples were collected from 198 persons, 198 at baseline review and at a varying number of reviews thereafter depending on when participants entered the study (mean 5.3 reviews per person, range 1-7), and 70 collected after a reported relapse. A total of 103 samples were collected within 45-days of reported relapse onset, 74 after relapse (57 within 30 days, 34 within 14 days and 16 within 7 days) and 29 prior (18 within 30 days, 8 within 14 days and 6 within 7 days). Of the 29 with

measures prior, 14 were also measured in the interval after relapse.

The association of anti-HHV-6 serological markers with other covariates was assessed using linear regression. All analyses were done using STATA/SE for Windows (Version 10.1; StataCorp LP College Station, TX USA).

8.4 Results

Table 8.1 shows the characteristics of the cohort. The majority of the cohort was female, had a RRMS type and moderate disability (median EDSS: 3.0) at study entry. There were 122 confirmed relapses during the study.

Table 8.1. Characteristics of 198 persons with clinically-definite multiple sclerosis in MS Longitudinal Study cohort

	n/N (%)
Sex	
Male	61/198 (30.8)
Female	137/198 (69.2)
MS type	
RRMS	149/198 (75.3)
SPMS	40/198 (20.2)
PPMS	9/198 (4.6)
Using interferon-β therapy	
No	84/198 (42.4)
Yes	114/198 (57.6)
	Mean (SD; range)
Age at study entry (years)	48.2 (11.4; 21-77)
MS duration from symptom onset at study entry (years)	14.0 (10.3; 0.1-57.6)
MS duration from diagnosis at study entry (years)	9.2 (8.8; 0-43)
EDSS at study entry	3.7 (2.3; 0-9)
MSSS at study entry	4.5 (2.7; 0.1-9.9)

Abbreviations: RRMS, relapsing-remitting MS; SPMS, secondary-progressive MS; PPMS, primary-progressive MS; EDSS, expanded disability status score; MSSS, Multiple Sclerosis Severity Scale.

Anti-HHV-6 IgG was detected in all baseline samples (mean titre: 216.1, median 160.0; range 10-640) (Table 3). There was no significant difference in the serum HHV-6 IgG titre by sex (p=0.210), age (p=0.971), disability as measured by EDSS (p=0.848), disease duration from symptom-onset (p=0.616), or course of MS at study entry (p=0.733) or exit (p=0.861).

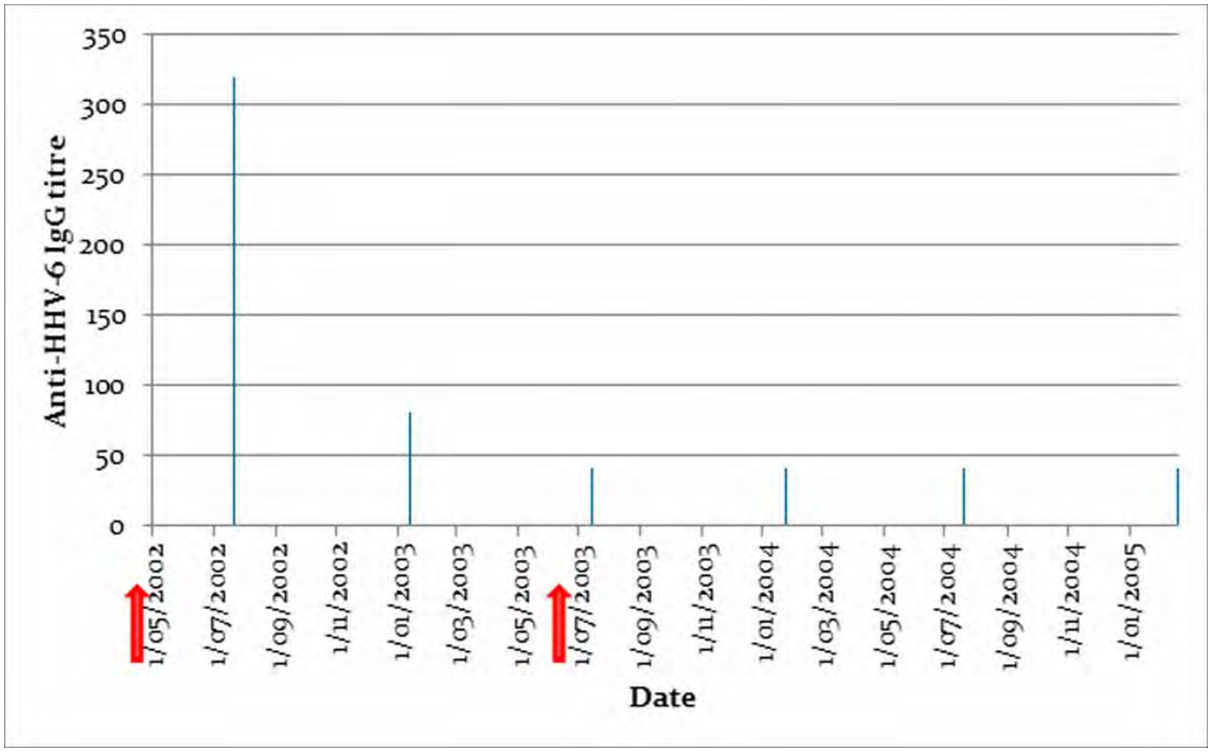
Table 8.2. Distribution of titres of serum anti-HHV-6 IgG and IgM

Baseline anti-HHV-6 IgG		Anti-HHV-6 IgM	
Titre	n/N (%)	Titre	n/N[1] (%)
<10	0/198 (0.0)	<20	1044/1050 (99.4)
10	11/198 (5.6)	40	4/1050 (0.4)
40	58/198 (29.3)	80	1/1050 (0.1)
160	90/198 (45.5)	320	1/1050 (0.1)
640	39/198 (19.7)		

[1] All samples positive for HHV-6 IgM were from one person, the remaining 1044 samples coming from the other 197 study participants.

Anti-HHV-6 IgM was measured in 1050 samples. All were below the 1:20 IFA detection threshold except for 6 (all from one person), which were elevated (Table 8.2). The HHV-6 IgM titres detected in the samples of the one HHV-6 IgM seropositive person declined over the study, starting at a titre of 320 at baseline, declining to 80 at the subsequent review, and remaining at 40 at each of the 4 remaining reviews (Figure 8.1). This person reported having a relapse 1.5-months prior to the high HHV-6 IgM titre measurement. A subsequent relapse that occurred during the study was not associated with a similar elevation. This person also had high tires of anti-HHV-6 IgG (640) at baseline. This person, a 51-year old female of mild disability (EDSS at study entry=2.0), with RRMS of 6 years duration at baseline who did not progress to SPMS during follow-up and was using interferon-β therapy, was not extraordinary from the cohort in any of these elements.

Figure 8.1. Temporal relationship of relapse (arrows below x-axis) relative to titre of HHV-6 IgM (bars above x-axis) for subject 73



8.5 Discussion

In this well-validated and characterized population-based longitudinal study of 198 people with MS, where people were assessed on average 5.3 times over an average of 2.4 years (1050 samples in total), we found virtually no occurrence of HHV-6 reactivation as assayed by serum HHV-6-specific IgM, even though 100% of participants were HHV-6-IgG seropositive at study entry and 122 clinically-definite relapses were recorded during the study. While this does not rule out a role for HHV-6 in MS, the almost complete absence of detectable anti-HHV-6 IgM (6 of 1050 samples), which is well below that which might be expected by chance alone, is not consistent with the notion that HHV-6 reactivation is associated with the clinical course of MS

We only had one person with detectable serum anti-HHV-6 IgM, with a pattern of HHV-6 IgG and IgM titres indicative of a reactivation of latent HHV-6 infection. While this reactivation may correlate with

the occurrence of a reported relapse 1.5 months prior to study entry, a second relapse occurring roughly 1 year into the study was not associated with an increase in HHV-6 IgM titre. Importantly, there were no elevations in serum HHV-6-specific antibodies suggestive of HHV-6 reactivation amongst any of the other 69 persons experiencing relapses during the study and where measurements were taken at variable distances from the relapse. For example, 16 samples were taken within 7 days of the reported relapse onset, 34 within 14 days, 57 within 30 days and 74 within 45 days. While theoretically some samples may have fallen back below the detection level by the time we measured the level, elevated and detectable anti-HHV-6 IgM titres have been found to remain high for a sustained period as long as several months(18, 19). The elevated titres of the person with high HHV-6 IgM titre waned but remained elevated for the duration of the study. We also had samples prior to the onset of some relapses (6 within 7 days, 8 within 14 days and 18 within 30 days), which is important as the onset of a relapse may be gradual and difficult to define and theoretically, reactivation may occur prior to a relapse. We had samples for most (74/122 (60.7%)) but not all relapses within 45 days, leaving the possibility of having missed some elevated HHV-6 IgM titres. Thus, while it is possible that we have missed some reactivations, the fact that only one out of 198 persons and 6 out of 1050 samples were elevated, argues against an association between HHV-6 IgM titres and relapses and progression of MS.

These findings are unlikely to be the result of inaccurate measure of relapse, given the rigorous quality assessment noted in Methods. Also, our findings of significant associations with season(15) and vitamin D(16) are consistent with findings elsewhere. While this does not rule out an association with subclinical exacerbations which would only be detectable by gadolinium-enhancing lesions on MRI, which we did not assess, the paucity of detectable anti-HHV-6 IgM makes a Type 2 error of this sort improbable.

Studies evaluating the relationship between HHV-6 reactivation, as measured by HHV-6-specific IgM,

and clinical course in MS are few. Chapenko and colleagues(7) evaluated a subset of patients in relapse in whom there was no detectable serum HHV-6 load, finding 2/6 to have detectable serum anti-HHV-6 IgM. Villoslada and colleagues(10) evaluated a cohort of 98 MS cases cross-sectionally, equally divided between RRMS and progressive courses, finding that higher HHV-6 IgM titres correlated with a lower EDSS ($p<0.0001$) and disease duration ($p<0.0001$).

The majority of studies evaluating the relationship between clinical course and HHV-6 reactivation have done so using HHV-6 load. We did not use viral load as a measure of HHV-6 reactivation in this analysis. Viral load can fluctuate within days and can therefore be easily missed if not sampled frequently. The wider detection window for serological markers relative to viral load seems therefore an advantage when sampling less frequently, as was the case in this study. HHV-6 IgM and viral load tend to correlate(20-23). Studies that examined associations between HHV-6 viral load and MS course have had equivocal findings, some(12, 24) finding no difference between relapse and remission samples, while others found higher proportions of samples with detectable HHV-6 load(5-7) or in the viral load levels(8, 13) during relapse compared to remission.

It has been suggested that anti-HHV-6 IgG is a better correlate of MS clinical activity than is anti-HHV-6 IgM. However, others have found no significant difference in anti-HHV-6 IgG between MS cases over various stages of disease(7, 10, 25, 26), nor over time in prospective studies(27), instead finding significant differences only in anti-HHV-6 IgM(10). Therefore anti-HHV-6 IgM may be a more specific marker of HHV-6 reactivation than variations in HHV6 IgG. While a significant increase in anti-HHV-6 IgG, along with detection of anti-HHV-6 IgM, are both in evidence during reactivation of latent HHV-6 infection(28), an increase in IgG titre may be reflective of a non-specific immune stimulus, whereas the appearance of IgM is more reflective of a specific antigen response. Given the uncertainty regarding the role of HHV6 IgG, and the possibility that increased titres of serum antibodies may be

reflective of overall immune activation, rather than specific response to HHV-6, we elected to measure IgG at baseline and HHV6 specific IgM serially as a measure of potential reactivation.

Overall, our findings add to a growing pool of research concerning the role of anti-HHV-6 IgM in MS. Previous work regarding HHV-6 reactivation has been conflicting and there has been a paucity of studies evaluating HHV-6 IgM and MS clinical course. Making use of the largest cohort study examining the role of HHV-6 yet undertaken, we have found little occurrence of HHV-6 serological reactivation, despite a fairly clinically active cohort. These findings are not consistent with a role for HHV-6 serological reactivation in MS clinical course.

8.6 Summary

Background: While human herpesvirus 6 (HHV-6) has been linked to multiple sclerosis (MS) aetiology, its relationship to clinical course is less clear.

Methods: Prospective cohort of 198 persons with clinically-definite MS living in Southern Tasmania over 2002-5. Serum anti-HHV-6 IgM was measured at each biannual review and at the time of relapse; serum anti-HHV-6 IgG was measured at baseline.

Results: Serum anti-HHV-6 IgM was measured in 1,050 samples from 198 subjects. While anti-HHV-6 IgG was detected in all subjects, only 6 samples, all from one subject, had detectable anti-HHV-6 IgM. While the initial sample, of the highest titre (320 units/mL) was measured relatively soon after a relapse (approx. 1.5 months prior), a subsequent measure days after another relapse was not elevated beyond the levels of the three preceding biannual measures (40 units/mL).

Discussion: Using the largest cohort of subjects and samples yet applied to the evaluation of the relationship of human herpesviruses and MS clinical course, we have found no association between HHV-6 serological reactivation and MS clinical course. This study does not preclude a role for HHV-6 reactivation within the central nervous system which may not be detected using serum. However, a role for a systemic HHV-6 reactivation in mediating MS clinical course is not supported by our findings.

8.7 Postscript

In contradistinction to the most ready interpretation of the results of Chapter 7, this cohort did not have any evidence of frequent reactivation of HHV-6 during the study interval, with only one person having detectable serological markers of reactivation. While this person had the highest titre of anti-HHV-6 IgG (640), none of the other persons with this titre had any evidence of serological HHV-6 reactivation. Similarly, the person in whom anti-HHV-6 IgM was detected showed no consistent association with relapse occurrence, nor was she of any abnormal level of disability. Thus, this analysis suggests that the observed association between anti-HHV-6 IgG and relapse and MS clinical course of Chapter 7 is not mediated by peripherally-detectable HHV-6 reactivation, though this does not rule out reactivation that cannot be detected with peripheral serology, including reactivation in the central nervous system.

8.8 References

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Chapter 9. Conclusion

Multiple sclerosis is a complex disease which, to the frustration of the biomedical research community, as yet refuses to give up much of its secrets. Work is thus still underway to discover why it occurs, why its clinical course plays out differently between patients, and what factors modulate both occurrence and clinical course. The analyses presented in this thesis are a fractal of the greater research community's work into understanding multiple sclerosis, approaching its study from a variety of angles, making use of a variety of methodologies on a range of study areas, in the hopes of making more sense of the greater whole. From the work presented in this thesis, some key conclusions may be drawn, along with their import on MS suffers today, as well as future research.

9.1 Geoepidemiology of MS

The first two analysis chapters focus on the geoepidemiology of MS, one looking in great detail at the change in MS frequency and distribution at the local level in the Greater Hobart region of Tasmania over time, while the second focuses on one aspect of MS epidemiology, its prevalence, over time and space across the world. The populations studied were necessarily disparate, one making use of primary data collected in a single study site, for part of the study duration actually making use of the same prevalent cohort, while the latter meta-analysis made use of data collected from the far flung corners of the earth, with many thousands of patients from a variety of populations. Similarly, the methods employed were varied, with one making use of relatively simple Poisson regression, while the meta-analysis made use of time-adjusted, log-transformed, random-effects meta-regression. Despite the great differences between these studies, they ultimately share in common the nature of the question each seeks to answer: what is the distribution of MS in time and space? In contrast to the later analysis chapters, which seek to evaluate determinants of disease, these first two chapters serve merely to establish the distribution of the disease in populations, local and global. However, it is studies of this nature that form the bedrock from which many of the more involved aetiological analysis spring forth.

Indeed, it was from anecdotal studies which noted the higher frequency of MS in the northern latitudes that led to the subsequent investigations into latitude, sunlight and ultimately vitamin D as factors involved in MS aetiology. Similarly, the finding from other studies of a changing sex ratio over time, borne out in a near-significant trend in this analysis, is providing needed information for hypothesis generation as to its cause, including interactions between environmental factors and sex.

Moreover, it is from the studies such as the local analysis of MS epidemiology over time in the Greater Hobart area, that provide the information policymakers need for planning allocation of resources. The evidence presented in Chapter 2 for an increasing prevalence of disease, to some extent due to the increased longevity and decreased mortality, but also an increased incidence among Australian-born females, indicates that costs associated with treatment for prevalent cases, but also potentially costs associated with carer support for persons with increased disability, can be expected to increase over time.

The study of MS geoepidemiology globally in Chapter 3 provides strong support in favour of the latitudinal gradient hypothesis, suggesting that in contrast to some others' findings(1-3), the general trend to an increase in MS prevalence with increased latitude is a valid one. While local exceptions in Scandinavian and Mediterranean Europe demonstrate the complexity of MS and, along with the significant difference in the latitudinal gradient between European and non-European populations, shows the significant interaction between environmental and genetic factors in MS aetiology.

A key element to this meta-analysis which others have not yet recognised nor made use of is that of time correction. While others have noted some difference in the associations between MS frequency and latitude over time(4), none have yet attempted to take into account the well-noted increase in MS frequency over time in evaluating its relationship with latitude. In the absence of taking into account this change over time, systematic reviews of MS frequency vs. latitude will inevitably be biased toward the null, by virtue of the non-random distribution of prevalence studies over time and space, namely

earlier studies done at higher latitudes and more recent studies being done at lower latitudes. This analysis found the magnitudes of the latitudinal associations doubled on adjustment for prevalence year. While this analysis demonstrated a significant positive association between prevalence and latitude prior to time adjustment, some others'(1) analyses which failed to demonstrate a significant latitudinal gradient may have been due to a failure to take into account prevalence year.

The strong demonstration of a potent and statistically significant association between latitude and MS prevalence is important not merely for its own sake, but rather for its support for a role for personal UVR exposure and vitamin D in MS aetiology. The debate over the gradient hypothesis has been going on virtually since the first comprehensive review in 1964(5), with some arguing it reflects inter-study variability in methods or improper comparison between studies(2, 6), and others that the gradient itself is an artifact of selective migrations of susceptible populations(2, 7). Were systematic reviews of the sort which failed to demonstrate a significant latitudinal gradient left unchallenged to undermine support for an association between latitude and MS aetiology, work evaluating a role for personal UVR and vitamin D might be similarly challenged as possibly reflecting some other confounding effect. On the contrary, the finding reported here of a strong and significant association between MS prevalence and latitude provides further support for a role for UVR and vitamin D in MS, substantiating the independent findings from case-control and cohort studies for each in mediating MS risk and clinical course.

This is not to rule out a role for genetic and ethnic factors in mediating MS geoepidemiology, as well as other environmental factors, including infections, sanitation and nutrition. Certainly the findings of significant differences in the magnitude of the gradient between European-descent regions, with the strongest magnitudes in the UK, North America and Australasia, are in sync with the known higher frequencies of genetic risk factors in these populations(8). However, in contrast to recent findings which suggested genetic factors accounted for 60% of the latitudinal variation in MS frequency in

Europe(9), this analysis found they accounted for much less than this. As a meta-analysis of aggregate-level data, it was not possible to evaluate the probable interaction of genetic and environmental factors in mediating MS distribution with latitude, and local studies are required to evaluate this appropriately. However, this analysis would indicate that there is a much stronger role for environmental factors which vary by latitude in mediating MS latitudinal variation than for genetic factors.

9.2 Vitamin D in MS

As reviewed in Chapter 4, there is an increasing wealth of epidemiological evidence in support of a role for personal UVR exposure and vitamin D in mediating both MS onset and modulating its clinical course, including the work presented in Chapter 5. Vitamin D's role as a potent immunomodulator is increasingly well-recognised, being able to depress inflammatory immune cell activity, as well as stimulate anti-inflammatory and regulatory immune cell activity. As described in the latter section of Chapter 4, vitamin D may also have a role to play in either modulating or wholly mediating some of the strongly-associated covariates in MS onset and clinical course, including smoking, pregnancy, stress, and childhood and acute infections, and herpesvirus reactivation. For some of these factors, such as smoking and to a lesser extent stress, plausible pathways of effect have been suggested – tobacco contains a range of immunomodulatory and generally deleterious compounds(10) which could affect risk of MS and affect its course, and stress is an inherently complex manifestation of the CNS-endocrine interface and thus could affect neuropathology and the immune system(11). Other pathways are less clear, however – while acute infections, particularly respiratory tract infections, have long been associated with increased relapse risk(12), no one has been able to propose a likely pathway connecting transient exposure to benign pathogens in the oropharynx and upper respiratory tree to pathology within the immunologically-privileged CNS. Similarly, while a range of physiological changes come with pregnancy and the immediate post-partum period, no one has been able to specifically label any aspect of this which might correlate with the reduced relapse risk during pregnancy and the significant increase in relapse risk immediately after. Vitamin D provides a neat and relatively simple connection for each

of these pathways to MS. In some of these, such as acute infection and stress, vitamin D may be a common antecedent for these and increased relapse risk. In others, such as smoking and pregnancy, changes in circulating vitamin D and its metabolism may be a partial mediator of the observed effects, with smoking interfering with proper vitamin D metabolism, and the post-partum increase in relapse risk acting via the loss of placental vitamin D metabolism. In other instances, such as childhood infection and reactivation of latent herpesvirus infection, vitamin D may act in interaction with these agents, modulating the immunologic tolerance to childhood exposure to otherwise benign antigens, and acting to enhance immunologic response to herpesvirus reactivation, and herpesvirus proteins interfering with vitamin D activity(13).

The work presented in Chapter 6 suggests another possible mediation by vitamin D, suggesting that one of the primary therapies used in treating relapsing-remitting MS, interferon-β medication, may act in part via its effects on vitamin D. The mode by which interferon-β medication exerts its therapeutic effects on relapse risk in MS has long been uncertain(14). In its role as a cytokine released by immune cells, interferon-β acts to induce an anti-infection and anti-tumour state in target cells. However, while pharmacological interferon-β therapy clearly exerts this effect, with treated persons frequently having side effects of malaise, fatigue and other flu-like symptoms, this is hardly the desired outcome and indeed, is an implausible one in exerting an anti-inflammatory effect. Rather, it has been suggested that interferon-β may act by repressing inflammatory cytokine expression(15) and enhancing anti-inflammatory and regulatory cytokine expression(15, 16), inhibiting the migration and proliferation of T-lymphocytes (15, 17, 18), reduced antigen presentation(15, 18) and restoration of blood brain barrier integrity(19). While these are all plausible pathways of effect, they also overlap appreciably with known immunomodulatory effects of vitamin D. The work presented in Chapter 6 demonstrates that interferon-β therapy shows a strong, positive interaction with sun in yielding vitamin D, and further shows that interferon-β only exerts a protective effect against relapse in persons with supra-

sufficient vitamin D, while in persons without sufficient vitamin D, it is actually positively associated with relapse risk. It may be that interferon-β exerts a portion of its effects by enhancing vitamin D production, acting at the level of initial production of pre-vitamin D in the keratinocytes, or possibly further downstream, by enhancing expression of *CYP27A1* and 25(OH)D production, or depressing expression of *CYP24A1*, which catabolises 25(OH)D. In increasing circulating levels of 25(OH)D, more would be available for metabolism to $1,25(OH)_2D$ by immune cells in the peripheral immune system, as well as by astrocytes and microglia in the central nervous system, and allowing it to exert its immunomodulatory effects in these cells. That circulating levels of $1,25(OH)_2D$ did not seem to be affected by interferon-β therapy is not surprising, since systemic levels are more associated with bone metabolism and are tightly regulated by parathyroid hormone. Levels of $1,25(OH)_2D$ in the microenvironment around immune cells, or in the CNS, might be more detectable with local assays, including lymph node aspirates or measurement of cerebrospinal fluid, and these might be expected to show a stronger association with interferon-β therapy like that of 25(OH)D, than would systemic levels. The observed positive association between interferon-β and relapse risk in persons with low vitamin D may be a reflection of the pro-inflammatory effects of interferon – the aforementioned anti-infection and anti-tumour effects – and may indicate that the majority of the anti-inflammatory effect of interferon-β therapy acts via vitamin D. In the absence of sufficient sunlight to generate vitamin D, regardless of the interferon-β enhancement, levels of vitamin D would remain low, and without its immunomodulatory effects, interferon-β therapy would solely realise pro-inflammatory effects, increasing relapse risk.

The works presented in Chapters 5 and 6 are strongly indicative for a role for vitamin D in modulating MS-related immunopathology, and provide support for use of vitamin D in treating MS. For both, however, randomised controlled trials are indicated to fully validate the observed effects of vitamin D, and to determine what treatment regimen is most effective in treating MS. While the work in Chapter

5 suggests that raising circulating 25(OH)D by 50 nmol/L could halve relapse risk, the work of Chapter 6 suggests that vitamin D is only effective against relapse in persons on interferon-β therapy. This effect may be due to the nature of vitamin D's source in this cohort, principally from sunlight exposure, with only minimal dietary and supplement intake. Thus, that only persons on interferon-β therapy showed an inverse association between vitamin D and relapse risk may reflect that only in these persons was the UVR-based vitamin D production pathway sufficient to yield therapeutic levels of vitamin D. If interferon-β therapy largely acts at the level of the keratinocyte production of pre-vitamin D, rather than downstream at 25(OH)D production and catabolism, then vitamin D supplementation could exert immunomodulatory effects in the absence of interferon-β therapy. In this case, assuming a majority of the therapeutic effects of interferon-β on MS clinical course act via vitamin D, interferon-β therapy could be wholly eliminated, in favour of sufficient dosing of vitamin D supplementation. Given the cost of interferon-β therapy, as well as the attendant side-effects, this would be preferable for patients and medical practitioners, in favour of the relatively cheaper and generally side-effect-free vitamin D therapy. Randomised controlled trials are needed, wherein patients are randomised to varying doses of vitamin D, controlling for sun exposure and stratified by interferon-β therapy.

9.3 Human herpesviruses & MS clinical course

Among the strongest associations found for MS risk are exposure to two human herpesviruses, Epstein Barr Virus (EBV) and human herpesvirus 6 (HHV-6). The work looking at the connection of each, particularly HHV-6 to clinical course is less clear and consistent, with some studies finding strong positive associations, while others find no significant association. All of these studies, however, have utilised a cross-sectional approach, either in analysis type or study design, evaluating anti-HHV serology titres and/or measures of viral load between relapse and remission samples, or in samples from persons with higher levels of disability relative to lower, and comparing the difference between. As yet, no studies have attempted to evaluate causation, with serological or viral load measures prior to outcome

being evaluated against outcome, either relapse or change in disability. The work presented in Chapter 7 is the first such study, evaluating baseline-measured anti-HHV-6 and anti-EBV IgG titre as a predictor of subsequent hazard of relapse, and the subsequent mean annual change in disability as measured by EDSS and MSSS. This analysis found a significant, dose-dependent positive association between baseline-measured anti-HHV-6 IgG and subsequent hazard of relapse, while no such association was found for either anti-EBV-EBNA or anti-EBV-VCA IgG. At the same time, no association was found for any of the anti-HHV IgGs and mean annual change in progression; however there was a significantly higher titre of anti-HHV-6 IgG among progressive course females relative to progressive course males.

There are a number of possible explanations for the association between higher anti-HHV-6 IgG and more clinical activity during the study. The most straightforward and simple link is that persons with higher anti-HHV-6 IgG have more frequent reactivation. While levels of anti-HHV-6 IgG hold relatively steady over time, making them a useful diagnostic marker of lifetime exposure to HHV-6, a recent reactivation of HHV-6 would be expected to induce a new immune response, which would induce a further raising of the anti-HHV-6 IgG. This in mind, the MSL study measured the serological marker of HHV-6 reactivation, anti-HHV-6 IgM, at each biannual review. As described in Chapter 8, however, there was a surprising paucity of samples in which anti-HHV-6 IgM was detected, only 6 out of nearly 1,050 samples measured, all of these from one person. In contrast to the diagnostic anti-HHV-6 IgG, which should hold steady levels over time, there is a visible decline in the levels of anti-HHV-6 IgM over the course of the study measures in the person with detectable levels, this in keeping with a reactivation of HHV-6 in the period immediately preceding entry into the study and no reactivations thereafter. This would suggest that if reactivations were causing the high levels of anti-HHV-6 IgG among persons with high disease activity, these reactivations were not detectable by measuring peripheral serology and may be occurring in the CNS.

Were an intra-CNS reactivation occurring, there are abundant fashions by which HHV-6 could manifest in pathological, neurotoxic effects, both direct and indirect(20). The most simple direct effect is a lytic reactivation in any or all of the glial cells in the CNS within which herpesviruses can establish latency, including astrocytes(21), microglia(20), and most relevant for MS, oligodendrocytes(22). A lytic viral cycle within any of these cells would result in a local inflammation within and around the site of reactivation, which could conceivably yield the inflammatory lesions typical of MS. In the course of entering cells, HHV-6 binds to the CD46 protein, found on immune and glial cells(23), inducing the expression of pro-inflammatory cytokines, including IL-1-β and IL-17(24). Moreover, HHV-6 encodes a viral analogue of a CCR2 ligand, which serves as a monocyte chemo-attractant(25), further enhancing local inflammation.

Beyond these direct effects, HHV-6 can transactivate the full or partial expression and replication of other herpesviruses(26), including EBV, possibly including the aforementioned viral IL-10, which could manifest in further local immune dysfunction and inflammation. Beyond this, however, HHV-6 can transactivate expression of human endogenous retrovirus proteins(26, 27), which, while not yielding fully functional virions, can yield expression of highly neurotoxic and inflammatory HERV proteins(28, 29). These pathways are not mutually exclusive, and indeed, all could act in parallel to yield the neuroinflammation attendant with exacerbations in MS.

An alternative possibility to account for the high levels of anti-HHV-6 IgG is that persons with the highest titres have a more vigorous immune response against the HHV-6 antigens. It is tempting to ascribe the high titres to a general immune hyper-reactivity, which would not be unreasonable in MS, an autoimmune condition defined by inappropriate inflammatory immune response. However, the absence of a similar association between the anti-EBV IgGs and disease activity would argue against such an interpretation. Rather, this seems to be a specific effect against HHV-6 antigens, or more likely so as

to manifest in neuroimmunopathology, host antigens in the CNS sufficiently similar to HHV-6 antigen as to elicit an autoimmune cross-reactivity(30, 31).

These findings are highly suggestive for some role for HHV-6 in contributing to the acute exacerbations of disease in MS; however there seems to be no evidence for a link between either HHV-6 or EBV and change in disability. At the same time, however, there was a novel difference by sex in titres of anti-HHV-6 IgG, with progressive-course females having significantly higher titres than their counterpart males. There was no association between anti-HHV IgG and progression from RRMS to SPMS during the study. Neither was there any difference in the relationship between anti-HHV-6 IgG and relapse hazard by sex, nor was there any evidence for a stronger trend, however non-significant, between anti-HHV titres and change in disability by sex. The absence of any consistency in these other parameters of clinical course may indicate the observed difference in anti-HHV-6 IgG titers by sex may be a statistical artifact. However, the well-known differences by sex in various aspects of MS, including differential association between incidence of disease and latitude(32) and the changing sex ratio over time(33), as well as the basic excess of progressive courses among males relative to relapsing-remitting courses among females(34), allow the possibility that this is a genuine effect.

9.4 Final conclusions of PhD

This thesis covers a variety of subject areas in MS research, including local and global geoepidemiology, as well as environmental and infectious predictors of clinical course. However, they make use of a semi-static local population in a high-prevalence area, and from this some significant conclusions regarding MS have been drawn including:

- Hobart continues to have the highest prevalence and incidence in Australia, with increasing prevalence increasingly being driven by an older and more aged cohort.
- This is in keeping with the well-known but long-debated latitudinal gradient hypothesis, which is strongly supported by the analysis of Chapter 3, finding a potent positive

association between MS prevalence and latitude, with local exceptions attributable to local idiosyncrasies.

- The latitudinal gradient is further supportive of a role for personal UVR exposure and personal vitamin D status in MS aetiology and its clinical course. As described in Chapter 4, vitamin D is a potent immunomodulator which is strongly linked to both MS onset and clinical course, and may in fact be a mediating factor in a number of other known pathways in MS onset and course.
- The work in Chapter 5 shows that personal vitamin D status, as measured by the diagnostic metabolite 25(OH)D, has a strong, inverse association with subsequent hazard of relapse.
- The most frequently-prescribed treatment for relapsing-remitting MS, interferon-β, may act in part by its positive interaction with personal sun exposure in yielding vitamin D , and indeed, requires sufficiency of vitamin D to manifest in therapeutic effects, the absence of which makes interferon-β therapy deleterious to MS clinical outcomes.
- The diagnostic measure of HHV-6 exposure, anti-HHV-6 IgG has a potent, positive association with subsequent hazard of relapse, this effect specific to HHV-6, but not acting via peripherally-detectable serological reactivation of HHV-6 as measured by anti-HHV-6 IgM.

9.5 Future directions

The MS Longitudinal Study cohort has accumulated a wealth of questionnaire and biological specimen data, much of which has yet to be evaluated. From this can and will be derived analyses regarding:

- The association of vitamin D with change in disability over time. Some studies have suggested a potential role for vitamin D and reduced disability by EDSS(35). This study is cross-sectional in design and thus, reverse causality is a major concern. With the MSL dataset, it would be possible to evaluate vitamin D measured prior to disability measure,

allowing a better assessment of whether a true effect is present, adjusting for baseline-measured disability and other relevant covariates.

- The association of viral load measures of HHV-6 and EBV on relapse and change in disability. The analyses in Chapters 7 and 8 are to some extent in conflict with preceding literature, which has shown a significant association between viral load measures of reactivation and relapse(36-40), as well as studies which showed a significant association between measures of reactivation and disability(41). The finding that virtually no reactivation of HHV-6 occurred during the study is somewhat surprising, particularly in light of the potent association between relapse and HHV-6 found in Chapter 7. Thus it will be useful to examine if a similar paucity of HHV-6 viral load is found and whether any association can be gleaned between viral load measures of reactivation and clinical course.
- The associations of serological and viral load measures of HHV-6 and EBV on MRI measures of clinical activity. Farrell and colleagues(42) demonstrated a significant association between serological markers of EBV exposure and MRI-measured parameters. The analysis of Chapter 7 failed to find any association between EBV serology and relapse and this may be due to some extent to the relatively mild disease in our cohort, which might have more subclinical activity detectable by MRI than clinically-evident relapse. Thus, it will be useful to assess whether any association is present between EBV serology and viral load and MRI-determined activity, and whether the HHV-6 and relapse association is borne out with MRI.
- The associations of personal UVR exposure and vitamin D on MRI measures of disease activity. The aforementioned study by Weinstock-Guttman(35) showed a significant association between vitamin D and reduced MRI activity. Other studies too have shown linked between vitamin D and season/UVR and MRI activity(43, 44). Here again the

prospective cohort study design at hand will allow a more definitive assessment of the link here.

- And a range of questionnaire-determined covariates on relapse, change in disability and MRI measures of clinical activity, including covariates related to physical activity, diet, medications, childhood infections, immunisations, chemical exposures, animal exposures, and various pregnancy and other women's health-related factors.

And so ends this thesis. The work presented here, a condensation of over three years of research by myself, and several years of work preceding my arrival developing and implementing the MS Longitudinal Study, will no doubt be of some utility to the field of MS research, and lays the groundwork for additional work to be completed in the years to come by myself, and others at the Institute.

9.6 References

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Appendix A: Other publications during PhD

van der Mei I, **Simpson, Jr. SL**, Stankovich J, Taylor B. "Individual and joint action of environmental factors and risk of MS", *Neurologic Clinics* (Invited review). May 2011; 29; 233 – 255.

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